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Design, challenges, and the potential of transcriptomics to understand social behavior

Wen Kin LIM[a] and Ajay S. MATHURU [ID] [a,b,c,*]

[a]Science Division, Yale-NUS College, 12 College Avenue West, Singapore, [b]Institute of Molecular and Cell Biology (IMCB), 61 Biopolis Drive, Singapore, and [c]Department of Physiology, Yong Loo Lin School of Medicine (YLL), National University of Singapore, Singapore

*Address correspondence to Ajay S. Mathuru. E-mail: ajay.mathuru@yale-nus.edu.sg.

Abstract

Rapid advances in Ribonucleic Acid sequencing (or RNA-seq) technology for analyzing entire transcriptomes of desired tissue samples, or even of single cells at scale, have revolutionized biology in the past decade. Increasing accessibility and falling costs are making it possible to address many problems in biology that were once considered intractable, including the study of various social behaviors. RNA-seq is opening new avenues to understand long-standing questions on the molecular basis of behavioral plasticity and individual variation in the expression of a behavior. As whole transcriptomes are examined, it has become possible to make unbiased discoveries of underlying mechanisms with little or no necessity to predict genes involved in advance. However, researchers need to be aware of technical limitations and have to make specific decisions when applying RNA-seq to study social behavior. Here, we provide a perspective on the applications of RNA-seq and experimental design considerations for behavioral scientists who are unfamiliar with the technology but are considering using it in their research.

Key words: behavioral variability, behavioral plasticity, neurotranscriptomics, RNA-seq, social behaviors

Next-generation sequencing technologies have had a major impact on medicine and biology in the last decade. They are being applied to the study of whole genomes to detect polymorphisms in a population, exomes to detect allelic variants in coding regions, and transcriptomes to detect spatiotemporal kinetics of gene expression in tissue. As these technologies have matured, their accessibility has increased, their costs have reduced, and their applications have become broader. Their impact is being seen in many research fields. For instance, medical diagnostics that use RNA sequencing (RNA-seq) to understand the likely causes of disease are generating information that can change prognosis due to new knowledge of underlying biology. A study by Sweet et al. illustrates this point. The authors of this study demonstrate that they were able to predict the transcriptomic signatures to distinguish between 2 types of end-stage heart failure—ischemic and dilated. Their ability to make such a distinction is improving the chances of designing a suitable course of treatment that was previously difficult (Sweet et al. 2018). These

techniques have similarly had a large impact on many biological discoveries. For example, it has been used to systematically track gene expression changes across developmental stages to study age-dependent changes in many different human and nonhuman systems (Gerstein, Snyder and Wang 2009; Yu et al. 2014; Blakeley et al. 2015). These analyses have led to the identification of master regulators as well as identification of isoforms of expressed genes, which in some cases have even led to the discovery of novel therapeutic targets for intervention.

RNA-seq is also becoming increasingly popular in the study of complex behaviors. A major driving force to apply such analysis is to examine if the transcriptome can be associated with behavioral phenotypes or the "phenome" (Bengston et al. 2018). In general, RNA-seq applied to the study of social behavior can be divided into 3 broad categories of problems. First, what role does the huge diversity of cell types within the brain play in controlling a particular type of social behavior? Second, how do we account for and explain

changes in the behavior of individuals under different conditions (i.e., context-dependent plasticity in behavior)? Included in this problem is comparing gene expression in a specific brain region or in a specific subset of neurons at different time points of certain behavior: for example, before exposure, during exposure, and post-exposure to a sexual mate. Third, how do we account for the differences in the same behavior between individuals (i.e., *inter*-individual variation)? That is, quantifying and comparing gene expression between individuals who are at different ends of a spectrum for behavioral phenotypes to achieve a mechanistic understanding of why such differences exist. We use recently published studies as examples from each of these 3 categories to illustrate what such studies are able to conclude through RNA-seq technology.

Successfully implementing RNA-seq to solve these problems can be difficult for researchers who are unfamiliar with the technology or are looking to use it for nonmodel organisms. We introduce this topic to such behavioral scientists and highlight important technical problems that one needs to be aware of when designing experiments. We highlight key considerations for experimental design including pooling and biological sample replication, and discuss social behavior-specific design issues throughout. We also point to other reviews that tackle specific issues in greater depth along the way.

Diversity of Cell Types in the Brain and Applications of Single-Cell RNA-Seq

The newest and most powerful tool in the RNA-seq arsenal is single-cell RNA-seq (or scRNA-seq). scRNA-seq techniques have evolved rapidly due to technological innovations since 2009 when the first papers applying this method appeared (Tang et al. 2009). For an overview of the history and how this technology has changed, refer to a review by Kolodziejczyk et al. (2015). As the name suggests, this technique allows RNA expression analysis at the resolution of individual cells. In comparison, traditional RNA-seq known as the bulk sequencing method averages the transcriptional expression of all cells in a sample. Most recent developments involve massively-parallel transcriptional profiling. An example of how this is achieved is a droplet method that separates, barcodes, and digitally counts 3′RNA in 1,000 s of cells simultaneously and applies automated computation to cluster cells based on individual transcriptional profile. Thus, this method allows high-throughput studies to be completed within a matter of months (Zheng et al. 2017). Such a technique will continue to be highly suited to the study of the brain as the diversity and complexity of cell types and neurons in a vertebrate brain have not been fully mapped, even for animals with brains that are relatively smaller than those of humans ($\sim 10^6$ or 10^7 neurons compared to $\sim 10^9$ neurons). In fact, the number of neurons and glia in different vertebrate brains has only been tabulated explicitly in the last decade or so (Herculano-Houzel 2009; Bartheld et al. 2016). The functional diversity of neurons in brain regions is still largely uncharted territory as demonstrated by many ongoing large-scale projects (Erö et al. 2018; Attili et al. 2019; Gouwens et al. 2019; Hodge et al. 2019).

Studies applying scRNA-seq have also begun to contribute toward uncovering this complexity. The cellular diversity with implications on functional diversity has turned out to be vastly more complex than anticipated even for regions of the brain that have had a long history of studies in a given species. A recent study (Pandey et al. 2018) examined a set of brain nuclei called the habenulae that have been previously considered to have at least 2 to 4 distinct anatomical subdivisions (Hikosaka 2010). Even though the habenula occupies a single voxel in a 7 tesla fMRI image of humans (Lawson

et al. 2014, 2017; Gosnell et al. 2019), it controls the release of multiple neuromodulators including serotonin, dopamine, and norepinephrine. The habenulae are considered critical nodes in the expression of aversive behavior in vertebrates and have been implicated in several disorders of the brain in humans. This includes addiction (Viswanath et al. 2013; Velasquez et al. 2014; Mathuru 2018), mood regulation (McLaughlin et al. 2017; Lee et al. 2019), depression (Browne et al. 2018; Drobisz and Damborská 2019), schizophrenia (Zhang et al. 2017; Schafer et al. 2018), and panic disorders (Pobbe and Zangrossi 2008; Mathuru and Jesuthasan 2013; Mathis and Kenny 2018; Ma et al. 2019). As the habenulae are critical nodes in cognitive and motor behaviors, they are also considered central structures in the social decision-making network (or the SDMN; Bshary et al. 2014). However, whether each of the subnuclei is composed of a single type of neuron (classified based on the neurotransmitters expressed for example) or multiple types were not clear prior to the study by Pandey et al.

The study team examined the habenula in zebrafish larvae that have ∼1,500 neurons at this stage of development. In their study, they implemented scRNA-seq, alongside anatomical brain registration of the sequenced neurons. To achieve this, the team used a transgenic line that marks habenula neurons with a fluorescent protein. They sequenced ∼13,000 individual cell transcriptomes (of neurons sorted from 25 larvae), which accounts for a 4-fold coverage of the number of cells normally present in a pair of habenulae. The authors identified 18 different subtypes of neurons from these transcriptomes using cluster analysis and principal component analysis that could be defined on the basis of the expression of key markers. They then performed RNA fluorescence *in situ* hybridization with a selection of these markers to identify the spatial expression patterns. This was followed by computational registration of images so that multiple zebrafish larval brains could be morphed on a single reference brain based on total ERK immunostaining (Randlett et al. 2015). The authors report that they could consistently place each subtype of neurons on such an atlas (Pandey et al. 2018). Their results suggested that neurons previously considered to be a single population because of the neurotransmitter expressed and their anatomical location in the habenula are further distinguishable at the transcriptional level.

One criticism of such categorization is that the use of finer classification tools leads to smaller categories, ad infinitum that may or may not have functional relevance. However, the study found that the subtypes identified at the larval stage, such as a cluster expressing *igf2a*, *adra1d*, and *tacr2* or a cluster expressing the genes *aoc1* and *kiss1*, were retained as distinct subpopulations into adulthood. In most cases, they could nominate 3–7 markers for each subtype and examine expression profiles in larvae and adults. The potential functional role of each subtype or combination is a promising future direction of study that will help address this common concern. Nonetheless, one possible interpretation of their results is that each subpopulation of habenula neurons plays a different role in the SDMN operation to precipitate different, but related behaviors. To understand the implications more clearly, the next step will be to integrate the information from scRNA-seq in specific behavioral contexts and examine the gene regulatory network (GRN) to decipher how neural diversity and biochemical activity are coupled (Baran et al. 2017).

Another recent study that examined the hypothalamic preoptic region of rodents after the execution of different social behaviors utilized a similar strategy. They devised a new technique to register the physical location of each subtype of neuron called the multiplex error robust fluorescence *in situ* hybridization or MERFISH

(Moffitt et al. 2018). As in the example of zebrafish habenula above, after performing scRNA-seq and identifying markers associated with each cell type, the authors then localized key markers back on to the brain tissue of other animals. In this case, the authors did not have a reference brain atlas, but by repeating the MERFISH experiment on multiple animals, the authors could tabulate the frequency of different cell types and their occurrence after the animal had engaged in different behaviors such as parenting or sex or aggressive territorial encounters. The authors of this study produced a remarkable report of 70 subtypes of neurons in the preoptic area and how different clusters of these subtypes are differentially activated in different sexes, during different social behaviors.

As is evident from the examples in this section, the main advantage of using scRNA-seq compared with bulk RNA-seq is in obtaining information on the different cell types present, which could in some cases provide an insight into the behavioral diversity. However, its applicability to the study of social behavior needs to be evaluated carefully on a case-by-case basis by the researcher. It is suitable, for instance, if the goal of a study is to understand how specific categories of neurons (inhibitory neurons versus excitatory neurons) or specific cell types (neurons versus glial cells from the same brain region) respond in 2 different conditions or at different time points. scRNA-seq is better suited to address such questions as this information is much more indirect and difficult to infer from bulk RNA-seq experiments. It is also suitable when limited tissue sample is available, provided that it was well preserved immediately after collection or was collected from fresh tissue. However, in spite of an increase in accessibility of the technology from the initial stages (Tang et al. 2009; Sevilla 2013), performing such analysis is still prohibitively expensive for most laboratories. Methods based on droplets (Macosko et al. 2015) or 10× Chromium platform (Zheng et al. 2017) reduces the costs of sequencing significantly. Customized solutions for handling and capturing cells of interest such as microfluidic-based devices (Tan et al. 2013) also reduce costs to some extent, yet the majority of the cost pertaining to reagents needed to obtain high-quality RNA as well as for analysis of the data produced remains high. Another point researchers need to consider is that scRNA-seq data require specialized bioinformatics attention to analyze and interpret the data as they are inherently noisier than bulk RNA-seq data. The analytical tools for appropriate normalization, imputation, and differential expression discovery are available but require extensive customization. The following reviews detail current perspectives on the challenges as well as the potential solutions for the suitable use of scRNA-seq data (Chen et al. 2019; Vieth et al. 2019). Finally, scRNA-seq necessitates complete cellular dissociation before analysis and therefore loses information regarding the spatial location of the cells being analyzed. The spatial location and connectivity information critical in most studies to make meaningful interpretations when large, unmarked regions of the brain are being studied can thus be lost. Field biology researchers also need to consider whether such connectivity information is relevant for their interpretation of data. If it is, then a much larger sample collection is necessary at the onset to perform *in situ* based anatomical registration after scRNA-seq.

Given these, bulk RNA-seq is still a preferred method even though scRNA-seq can be applied to address questions of behavioral plasticity and individual variation described below. Particularly so, as researchers can perform experiments with a larger number of biological replicates or time points for similar costs.

Behavioral Plasticity

Comparative analysis of vertebrates shows that a shared SDMN in the brain regulates species-specific adaptive behaviors (O'Connell and Hofmann 2011). Researchers are often interested in applying RNA-Seq to characterize and compare transcriptional changes in these brain regions in organisms under different contexts to understand the molecular underpinnings of behavioral plasticity. For example, the scRNA-seq analysis of the preoptic region of rodents detailed above examined differential expression profiles after different social behaviors were performed. In a majority of other studies, researchers have focused on differences in the whole brain or brain regions in different contexts using bulk RNA-seq. Studies have analyzed gene expression changes in different brain regions in an animal that shows a seasonal change in the behavior as it often allows collection of an adequate number of samples showing comparable behavior. For example, 1 study compared free-living tree swallows *Tachycineta bicolor* in 2 conditions—when they are establishing and maintaining a territory to attract mates and when they switch to parenting (Bentz et al. 2019a). The authors found that expression in different brain regions previously associated with social behaviors changed differentially. The hindbrain and the hypothalamus showed a shift in the expression of a large number of genes involved in immune processes and glucocorticoid signaling, but far fewer changes were recorded in the third brain region of the ventromedial telencephalon. The authors interpret this result to be consistent with a change in physiology in response to different types of environmental stressors. In another study, RNA-seq was used to identify transcript changes in guppy brains and whole bodies that have been reared in the presence or absence of predators (Fraser et al. 2011). The use of whole-brain tissue limits the types of analyses that can be conducted when compared with the mate preference study (in the section below) which used tissue from specific brain regions. However, as the authors here examined lifetime changes in animals living with or without constant predation threat, they could identify habituation associated brain-wide changes.

It is interesting to note that this study was also completed before the full genome assembly of guppies was available, demonstrating how RNA-seq can be used in nonmodel organisms where full genome assemblies or reference transcriptomes have not yet been produced. The authors, in this case, used genome assemblies of other fish species (medaka and zebrafish) to predict transcripts of guppy fish. Another possibility in such a situation is to assemble a transcriptome de novo without the need to rely on genetic information from a related species as has been demonstrated in other studies (Vijay et al. 2013; Bentz et al. 2019b). The authors of this study, however, recommend using sister species assemblies when possible for higher reliability in the results (Vijay et al. 2013). To this point, a recent study demonstrated that the assumptions that de novo assembled transcriptome is unbiased approximation of relative abundance is often violated. So, studies adopting this approach should carefully evaluate their data and employ other practices that minimize any bias that may occur (Freedman et al. 2020).

These 2 studies highlight 2 key choices researchers studying social behavior have to make when designing a study. First, the appropriate time window to isolate the brain RNA after a social event, and second, the choice of specific brain regions. Obtaining an adequate quantity and quality of RNA from the sample is a common but critical technical challenge in all situations as seemingly minor changes in the time of collection or spatial difference in the dissection of the brain region can significantly affect differential

expression analysis. Studies that have examined multiple time points after a social interaction suggest that transcriptional profiles can change in waves over 30, 60, and 120 min (Petretto 2017; Srivastava et al. 2018; Bagnati et al. 2019). These waves could reflect different short- and medium-term events. Therefore, depending on the question of interest, researchers can focus on changes in the short term (10- to 30-min window), medium term (span of hours), or long term (over a lifetime).

Individual Variation in Expression of a Behavior

The third application of RNA-seq is to investigate the biological mechanisms that result in behavioral variation between individuals (i.e., *inter*-individual differences). A recent example is the study of honeybee response to parasitic mites (Diao et al. 2018). In this study, the researchers performed a genomic analysis of 2 sister species to understand the genetic differences associated with specific adaptations. In addition, they also compared the brain transcriptomes of individuals from the same species *Apis cerana* that exhibit different responses to parasitic mite infection, a key threat to their survival. After exposing bees to parasitic mites and monitoring their defensive response, the researchers categorized the bees into 2 groups based on the distinct defensive behavior exhibited—self-grooming (using their legs and pivoting abdomens to remove mites) and body-shaking. They then sequenced the whole-head transcriptomes. They found 502 genes were upregulated in grooming individuals and 48 in body-shaking individuals, while 11 and 60 genes were downregulated in the same groups, respectively. Among the genes whose expression profile changed, upregulated genes in grooming bees were enriched in DNA replication and repair functions while downregulated genes in both grooming and body-shaking bees were enriched in response to biotic stimulus (determined by Gene Ontology [GO] terms). Another example is the study of Trinidadian guppy *Poecilia reticulata*, a useful model for mate choice research. RNA-seq, in this case, was performed on specific brain regions to compare gene expression in females with distinct preference phenotypes (Bloch et al. 2018). Females with and without preference for colorful males were compared. Their results demonstrate that within 10 min after exposure to colorful males, guppy females that have clear preferences for colorful males have a distinct transcriptional profile both in the sensory processing area of the brain (the tectum) and in the decision-making region (the telencephalon). Their analysis of the RNA-seq data also revealed transcription factors that likely act as "neuromolecular switches" that trigger transcription of genes associated with mating decisions in individuals that have a mating preference.

These results suggest that the same environmental stimuli can elicit different endogenous processes related to distinct behavioral responses. The terminal nature of such experiments, however, makes it difficult to address questions such as the frequency and repeatability of an individual's behavior to the external stimulus and the accompanying transcriptional changes directly. It is also unclear exactly what steps connect the upregulated and downregulated genes to the behavior observed. A systems-level computational analysis with larger datasets of a similar kind could be the next step to understand the relationship between molecular function ontology and the overall phenotype (Petretto 2017; Srivastava et al. 2018; Bagnati et al. 2019).

Technical challenges in conducting RNA-seq experiments

Issues in isolating sufficient, high-quality RNA

The first crucial step in any RNA-seq experiment is the successful isolation of RNA from the tissue of interest and in many cases, isolating sufficient amounts of high-quality RNA is difficult. Regardless of the size of the organism being studied, RNA degrades rapidly so dissecting brain regions (or any other small regions of interest) and isolating RNA from these regions presents a technical challenge. For example, if dissections are being performed in brain samples from zebrafish, these dissections have to be conducted rapidly (within 2–3 min) without compromising the ability to demarcate unlabeled brain regions. For small animals, in particular, there is also an additional layer of difficulty in obtaining sufficient RNA. Only 1–2% of total RNA in a cell is messenger RNA (mRNA), which is the target of sequencing, whereas 90% of total RNA is usually ribosomal RNA which needs to be removed (Conesa et al. 2016). RNA must have both high purity (measured by a ultra violet (UV) spectroscopy absorbance ratio at 260 nm and 280 nm of at least 2.0) and high quality with an RNA Integrity Number (RIN) of at least 7 out of 10 with 10 representing the highest quality with least degradation (Sheng et al. 2017). It is important to note that RIN measures are standardized to mammalian organisms. Other species with abnormal ribosomal ratios, like insects, may generate poor RIN numbers (Kukurba and Montgomery 2015). Therefore, in studies on social behavior, researchers studying nonmammalian and nonmodel organisms need to pay close attention to the ribosomal ratios and subsequent RIN measures of their samples.

RNA degradation can severely damage the potential of sequenced data to accurately represent *in vivo* gene expression as the effects of degradation may not be uniform. Libraries constructed from low-quality RNA are compromised as they have fewer reads (Sheng et al. 2017) and these reads are of decreased complexity as low expression genes may be absent (Gallego Romero et al. 2014). Gallego Romero et al. compared RNA samples of peripheral blood mononuclear cells that had been left to degrade for increasing durations at room temperature. Comparing between high-quality RNA samples (with a mean RIN of 9.3 and extracted immediately) and degraded RNA samples (with a mean RIN of 3.78 and left to degrade for 84 h), the authors found that 61% of genes were classified as differentially expressed between the 2 groups (Gallego Romero et al. 2014). Additional risks of low-quality RNA are uneven gene coverage and 3'-5' transcript bias (Gallego Romero et al. 2014; Kukurba and Montgomery 2015). Given these risks, researchers should put great emphasis on accurately assessing RNA quality as all subsequent steps depend on the sample quality.

After sample isolation, ribosomal RNA (rRNA) should be removed for library preparation. Researchers have to make a choice here as 2 standard approaches are available—rRNA removal or poly(A)-tail selection. The rRNA removal approach includes methods like RNase H, where rRNA bound to DNA oligos is digested and then removed with DNase, and Ribo-Zero, where rRNA bound to RNA probes are attached to magnetic beads which are then subsequently removed. The benefit of rRNA removal is that it enables the detection of nonpolyadenylated transcripts and small RNAs. Poly(A)-tail selection involves using oligo(dT) primers to enrich for polyadenylated RNA transcripts that removes rRNAs as well as all nonpoly(A) RNAs (Zhao et al. 2014). The benefit of poly(A)-tail selection is that lower sequencing depth is needed.

For samples with limited RNA quantity or lower quality, as might be the case for samples collected in the field in behavioral studies, the rRNA removal approach, and the RNase H method, in particular, is recommended because it results in a lower mean coefficient of variation (CV) indicating less variation, higher coverage from 5′ to 3′ ends, and stronger detection of gene expression compared with poly(A)-tail selection methods (Adiconis et al. 2013; Schuierer et al. 2017). When the amount of tissue sample is a limiting factor, researchers can take advantage of methods optimized to extract appropriate quality and quantity of RNA from micrograms of tissue, such as extraction by laser microdissection (Farris et al. 2017; Nichterwitz et al. 2018).

If researchers are in the field or have other constraints such as costs and extraction of scarce tissue, they can design their experiments keeping 2 choices in mind. The first is whether or not to pool samples together for subsequent sequencing in their experimental design. The second is to decide on a trade-off between the number of biological replications and sequencing depth (the number of times a fragment is represented in a collection of sequences). Both of these choices can greatly affect the costs, interpretation of sequencing experiments, and the quality of results and are elaborated on below.

Issues with pooling samples

In a classical sense, pooling samples mean combining multiple individual samples before the analysis of differential expression. Pooling multiple individual samples together would constitute 1 library and hence function as 1 biological replicate. In contrast, not pooling at all means constructing a single library for *each* biological sample. One reason to pool is to reduce costs associated with sequencing each sample, but researchers may also pool samples due to limited tissue availability or low RNA quantity. Another reason some researchers use pooling is to overcome batch effects in sequencer performance (Wang et al. 2012). Todd et al. found that 63% (100 out of 158) of ecology or evolutionary biology studies between 2010 and 2014 pooled samples (Todd et al. 2016). Given how frequently pooling is applied, researchers should be aware that results from pooled sequencing data have to be interpreted more carefully because each pool is treated as a single sample and should not be confused with the total number of biological replicates.

Among the statistical issues highlighted as potential concerns, estimating differential gene expression and false discovery rates due to pooling bias have been described in detail (Rajkumar et al. 2015). Pooling bias is defined as the difference between the pool value and the mean of the samples measured individually on a log scale (Mary-Huard 2007). The pooled log transformation of samples can result in misleading biological averaging (Kendziorski et al. 2005). Second, there is also a lower within-group variance in pooled samples compared with the true within-group variances of individual samples (Balakrishnan et al. 2014; Rajkumar et al. 2015). However, it has also been argued that if only a limited number of libraries can be constructed, it is advantageous to pool samples from multiple individuals and sequence *multiple* pools to sample biological variability. This method is considered better at sampling variability than sequencing equivalent number of libraries prepared from single samples. Researchers are referred to Todd et al. for a discussion on the pros and cons of pooling applied to ecological samples to determine a suitable strategy for their experiments (Todd et al. 2016).

Researchers need to be aware though that the term "pooling" can also be used in a different manner—when multiple, individually prepared libraries are combined to be sequenced together in a single lane. In this case, individual samples are barcoded with a unique identifier sequence during the library preparation step before multiplexed sequencing (Figure 1). This is done routinely in most current RNA-seq studies to maximize the use of the larger than necessary depth of sequencing available in NextGen sequencers (detailed in the next section). Barcoded sequencing is also often performed to avoid batch effects. Batch effects are differences in measurement across separate procedural conditions that are unrelated to the biological or scientific variables in a study (Leek et al. 2010). Batch effects can also arise from the technical differences in sequencing lanes. Detailed analysis of pooling strategies and recommendations are, however, available to help researchers make choices suitable for their experiments (Wang et al. 2012).

Issues with the trade-off between the number of biological replications & sequencing depth

Researchers also have to make a decision on the sequencing read depth and the number of biological replicates needed as they affect both the cost and the power of the experiment in making genuine biological discoveries. The number of biological replicates is particularly important given the reproducibility crisis due to underpowered studies highlighted in psychology and neuroscience (Button et al. 2013; Ioannidis 2014; Cumming and Calin-Jageman 2016). In RNA-seq experiments, often a minimum of 3 biological replicates per condition is used. However, this number is arbitrary and many more replicates may be needed depending on the question of interest, the variance in the system being examined (Liu et al. 2014), as well as the statistical power that the researchers wish to achieve. This information may not necessarily be available to researchers, though it might be possible to estimate the number of biological replicates that will be necessary based on other experiments of similar nature. As this is case specific, researchers should examine the best practices that suit their experimental needs (Hart et al. 2013; Conesa et al. 2016).

Increasing the number of replicates at the highest depth possible may seem to be the most straightforward approach. However, that combination is neither practical nor sometimes necessary. Increasing the sequencing depth beyond a point has diminishing returns on the power to detect differentially-expressed genes (DEGs) as compared with increasing the number of biological replicates (Liu et al. 2014; Rajkumar et al. 2015). The cutoff point depends on the organism being studied and the number of transcripts expected in a tissue. For example, a bacterial sample will need a lower depth than what other organisms need (Haas et al. 2012). One study using the human cell line, MCF7, found that increasing reads from 10 to 15 million (a 50% increase) for 2 biological replicates only increased the average number of DEGs identified by 6% (Liu et al. 2014). The same study found that, in contrast, adding a 3rd replicate at 10 million reads increased identified DEGs by 35%. However, higher sequencing depth does have advantages in certain cases such as when informative transcripts are shorter or are expected to be rare. For rare transcripts with fewer than 10 fragments per kilobase of exon per million reads mapped, 80 million reads are needed to accurately quantify differences (Sims et al. 2014). The same study also estimated that more than 200 million paired-end reads are needed to detect a full range of transcripts, including isoforms in human samples (Sims et al. 2014). However, the cost of such experiments is prohibitive and should be chosen only if researchers have specific background information that suggests the need for highest depth. Especially as saturation is reached, increasing the number of reads does not necessarily translate to an improvement in detecting differentially-expressed transcripts (Tarazona et al. 2011). Practically, for
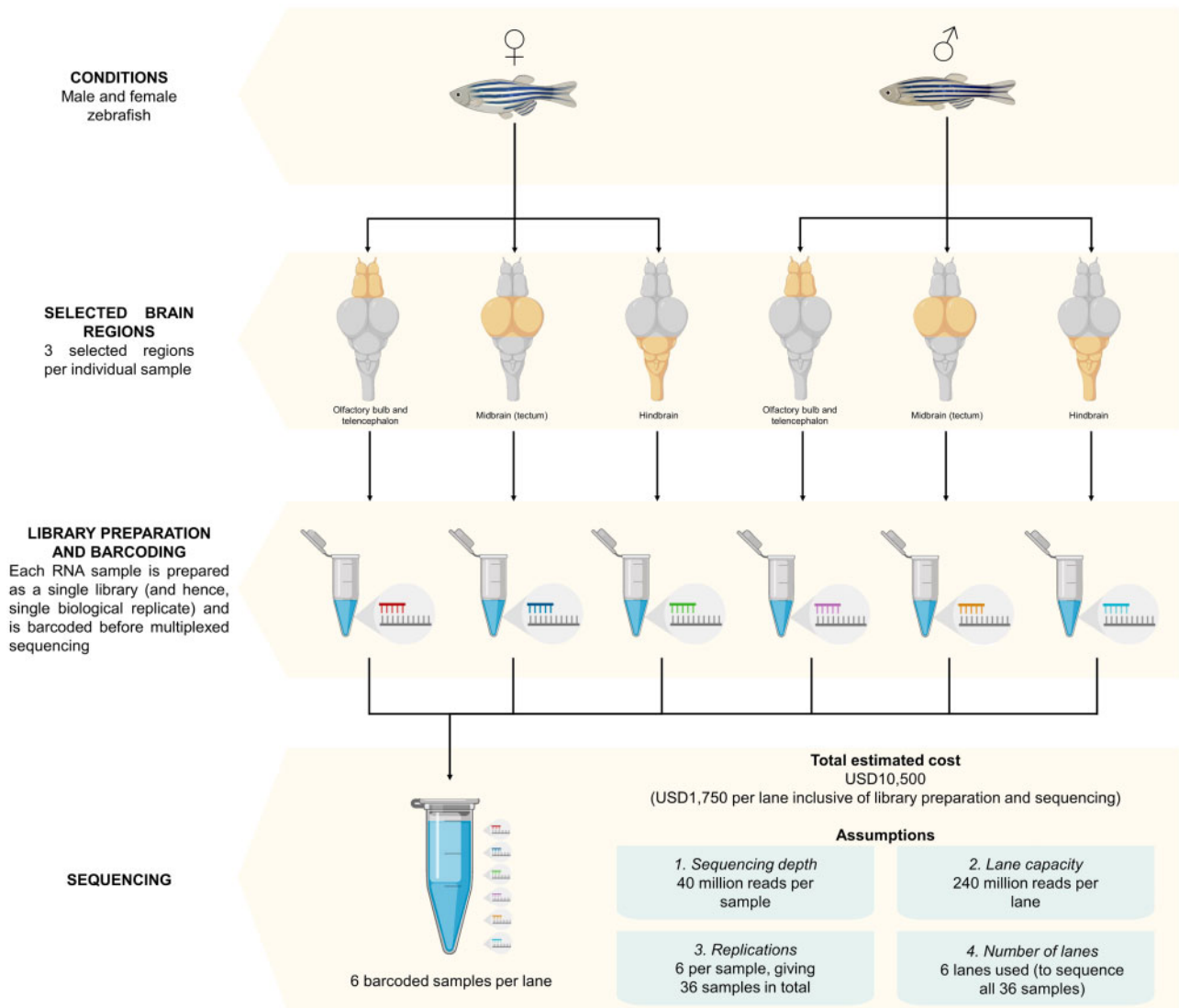
Figure 1. Schematic of an example RNA-seq experiment. The schematic shows a potential design to compare 3 brain regions between the 2 sexes in a simple social behavior experiment and the approximate costs for performing such an experiment with individual samples that are barcoded and sequenced as individual libraries.

most studies and organisms, this often translates to a depth in the range of 40–60 million reads (Conesa et al. 2016). In general, if multiplexed sequencing is being performed (Figure 1), researchers can opt for increasing biological replicates as it increases the power to detect DEGs regardless of sequencing depth. However, researchers need to decide on a balance in experimental design as increasing either biological replicates and sequencing depth adds notable costs.

Challenges in applying RNA-seq to the study of social behavior

It might have become evident to the reader as pointed out in each of the sections above that RNA-seq experiments pose a few unique challenges when applied to the study of social behaviors. Unlike quantification of changes in cells from any other part of the body such as heart tissue from a biopsy, there are 4 sources of dynamic changes that need to be taken into account in the study of social behaviors. First, social behaviors involve subjects as well as partners. Second is the problem of discretizing continuous, sometimes co-

occurring behaviors. Third is that multiple, discrete brain regions are involved in social behaviors such as the structures that form the SDMN (O'Connell and Hofmann 2011) and each region may change differentially. In addition to the brain regions of interest, experimenters may also need to sample other tissue such as the gonads, therefore increasing the total number of samples that need to be sequenced in an experiment. And finally, the temporal plasticity of gene expression in these brain regions during the course of the behavior is also a large variable.

An example of studying courtship behavior of sticklebacks below illustrates these 4 challenges. Animals in the wild often need to switch between courting the member of the opposite sex and aggression toward competitors (Sanogo and Bell 2016). Designing experiments that delineate these different behaviors, which are continuously modified depending on the reciprocity and partners present, require researchers to make decisions regarding the method used for classification of the behavior and identify what their study will be able to address. The interaction between the social partners

adds a dimension of variability that is not easy to control even after increasing the number of experimental replicates. Researchers, in this case, made a choice on the brain region of interest (diencephalon and cerebellum) in advance as taking larger areas of the brain averages differential expression profiles between conditions. However, these are still fairly large divisions with multiple SDMN nuclei within. Researchers would have had to devise optimal protocols that ensure the quality of the RNA obtained if they were opting to collect smaller brain regions by microdissection. If researchers use nonmodel organisms then information on the structure and organization of brain regions may not be available so strategies to collect brain regions in a consistent manner from different individuals are also difficult. The choice of time points, such as 30 min post an encounter with a novel female, is common but the results can be impacted by intermediate events which can increase the noise in the replicates. Finally, the main challenge is to collect an adequate number of samples under the same treatment conditions. This need is particularly acute if researchers aim to use scRNA-seq and expect to register the transcriptomes to a brain atlas as described above.

Recommendations

Key considerations before researchers pool samples

Since it is impossible to detect whether individual outlier samples are distorting the mean and median expression of a pooled group after pooling, our default recommendation for researchers is to sequence samples individually as this is statistically most powerful. However, researchers may consider pooling due to one of the reasons described above. Most common in social behavior studies or those collecting samples in the field being small segments of the brain or gonads used, or insufficient RNA from individual samples for sequencing. For reference, a single commercial library preparation in bulk RNA-seq will most often request the user for at least 1 μg of RNA for optimal results (with 0.1 μg being the minimum amount required depending on RNA quality) to generate adequate mRNA after rRNA removal and poly(A)-tail selection (for Illumina's TruSeq Stranded Total RNA and TruSeq Stranded mRNA protocols, respectively; Chao et al. 2019). If pooling is required to reach the threshold amount of RNA, then researchers must consider one additional factor to mitigate some statistical issues.

Researchers need to consider the likely sources of variability of gene expression in their experiment. Variation in gene expression can be attributed to 3 components—across group variability, measurement error, and biological variability (Hansen et al. 2011). "Across group variability" is the difference in gene expression between 2 conditions of interest and is what researchers are usually interested in determining. To be able to determine this, researchers need to minimize measurement error, the variance that arises from procedural differences in replicates. Biological variability is the inherent differences in gene expression between individuals and can also confound group variation results. As described above, pooling a large number of samples can be an effective strategy if researchers expect either high biological variability (as measured by CV or based on previous qRT-PCR experiments), or if they expect small differences between conditions to prevent bias of nonrepresentative outliers (Todd et al. 2016). However, researchers will need to remember that an entire pooled sample is considered 1 biological replicate. Therefore, if the goal of the researchers is to identify differential expression between different time points or 2 conditions (Figure 1), then they will have to design the experiment and sample collections

in such a manner that they are able to sequence multiple such pools to make reliable biological discoveries.

A pilot experiment

Our second recommendation is that researchers conduct a pilot experiment. This is useful to estimate the expected variability between biological replicates and to decide on optimal sequencing depth. In designing such an experiment, researchers can choose fewer biological replicates for the main conditions of interest and conduct multiplexed sequencing within a single lane in order to minimize costs. For example, a pilot experiment design might use a single NextGen sequencing lane which allows ∼240 million reads per lane. In these experiments, individual sample identity is determined by barcoding during library preparation before sequencing. Researchers can explore sequencing between 3 and 8 individual samples for 80 million or 30 million reads per sample, respectively. Thus, they can utilize the full 240 million read count of the lane minimizing the cost. Estimates based on recent quotes (November 2019) suggest that one such experiment can be currently conducted for approximately US$1750.

After the pilot, researchers can use the available tools to determine if sufficient read depth has been achieved or if additional reads are required. For example, Bass et al. developed the superSeq tool to model the relationship between statistical power and read depth that can be applied to a completed experiment in order to maximize statistical power (Bass et al. 2019). In addition to informing the optimal depth that is needed in the full-scale experiment, a pilot experiment can provide prototype data to estimate variance between biological replicates and determine how many replicates are required. Busby et al. developed Scotty, an online tool, which uses pilot data as inputs to estimate the optimal read depth and the number of replicates based on cost (Busby et al. 2013). Using these tools to determine the required sequencing depth reduces the Poisson variance which occurs in RNA-seq data when each particular RNA read is selected and counted at random (Busby et al. 2013). While the Scotty tool can also use publicly available datasets instead of pilot experiment data, pilot data generated under the same experimental conditions provides a more accurate prediction of power (Busby et al. 2013). Given the necessity of a pilot experiment, especially for nonmodel organisms where information about CV or optimal depth might not be known, researchers should only embark on RNA-seq if they have sufficient resources to run both a pilot experiment and a full-scale RNA-seq experiment. Without doing so, they risk facing many of the statistical issues in a full-scale experiment that could invalidate potential results.

Validation and further steps

Apart from the 2 recommendations above, researchers can go further with their RNA-seq data in a few ways. To start with, they can incorporate an additional measure to validate RNA-seq results by combining it with at least one other assay which tests for the same conditions. In most cases, this is achieved by performing qRT-PCR for a few selected markers identified in the RNA-seq. In other cases, researchers complement RNA-seq studies with other experiments, such epigenomic profiling, ChIP-seq, mass spectrometry-based proteomics, and additional behavioral assays. Multiple lines of evidence, especially a concordance between RNA analysis and the proteome, for example, provide greater confidence in interpretation.

Finally, researchers can apply new computational biology approaches that allow them to take a step further than quantifying
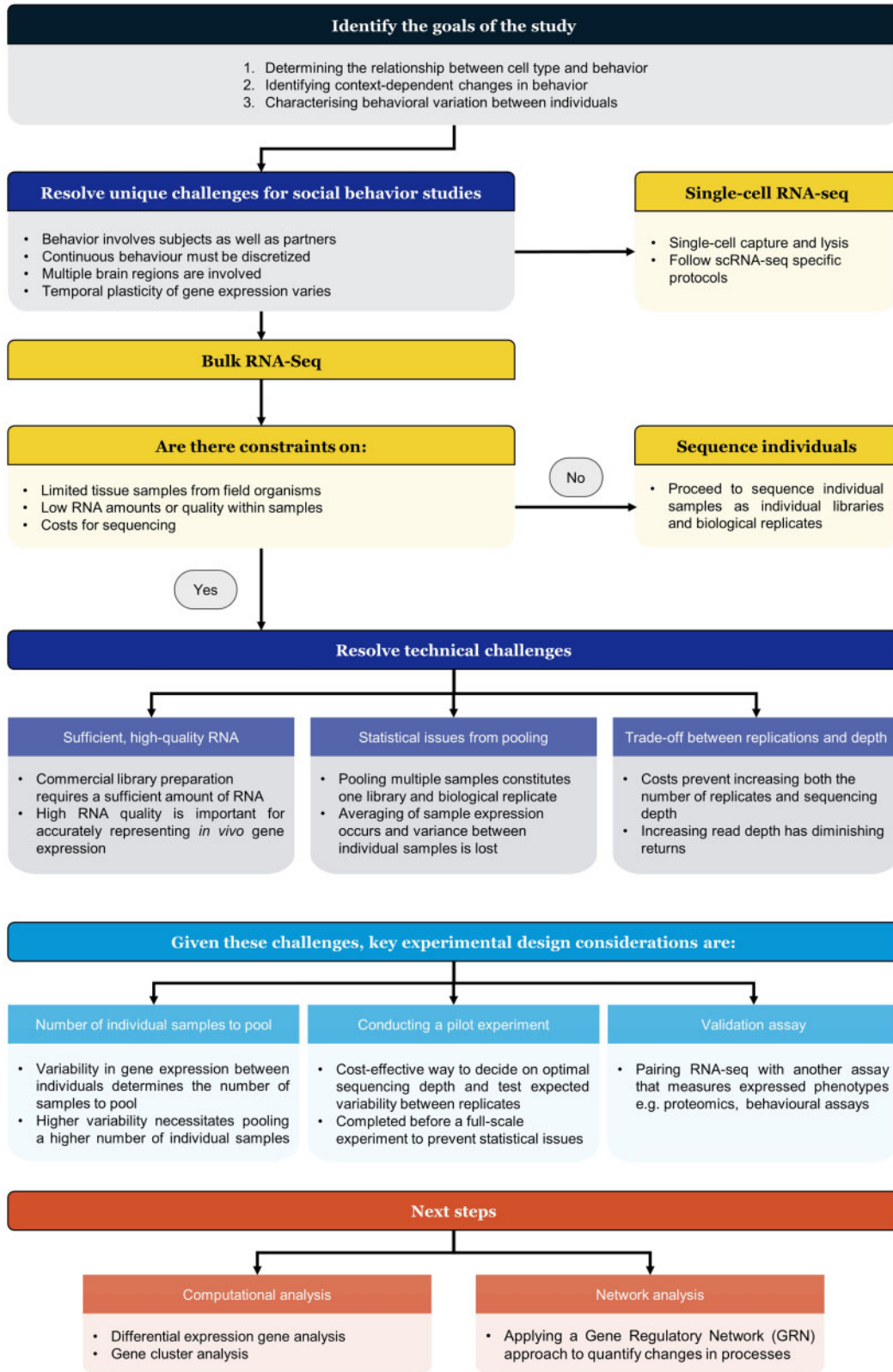
Figure 2. Flowchart of challenges and key considerations in experimental design. In designing a RNA-seq study, researchers must resolve challenges relating to social behavior studies and decide whether single-cell RNA-seq or bulk RNA-seq will best achieve the study goals. Given the technical challenges when faced with scarcity of RNA samples or quality, and cost constraints, researchers should consider the number of individual samples to pool, conducting a pilot experiment, and including a validation assay to ensure valid data from a RNA-seq experiment.

individual gene transcripts based on gene ontology terms in specific brain regions. Among them, a GRN approach noted above focuses on quantifying changes in pathways and biological processes in networks of neurons represented as nodes and edges in a network. Such an approach has emerged as a powerful method as systematic changes in the interconnected neural circuitry are examined between conditions (Baran et al. 2017). A systems-level bioinformatics approach that examines coexpression modules from the RNA-seq data and how these modules change overall between 2 conditions is a second new approach that researchers can apply to enhance the power of biological discoveries from RNA-seq data (Srivastava et al. 2018).

Conclusion

Overall, RNA-seq and transcriptomic technologies have great potential for understanding the mechanisms behind social behavior with the possibility of providing answers to long-standing questions on behavioral plasticity at the individual and species level in any organism. They also have the potential to unravel the interaction between environmental stimuli and genetics. Figure 2 gives an overview of the challenges and key considerations in designing RNA-seq experiments. In order to make use of the powerful technology in a cost-effective and feasible manner to produce valid results, researchers must carefully design experiments and be aware of the trade-offs, risks, and benefits inherent in experimental design with pooling, replication of samples, and a pilot study.

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