Cell-specific transcriptional analysis in complex tissues

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Abstract

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Cell-type specific transcriptional analysis can yield important information about the role of a certain cell type in the context of its tissue microenvironment. Previous methods for isolating cell type-specific transcriptional data have included enzymatic or mechanical tissue dispersion followed by magnetic cell sorting (MACS) or fluorescence-activated cell sorting (FACS) and laser capture microdissection. However, these methods have important limitations. Transcriptome analysis by translating ribosome affinity purification (TRAP) allows the researcher to isolate translating mRNA from a genetically defined cell type. Discussed in this review are current methods for translating ribosome affinity purification, caveats of this method, and considerations for successfully completing these studies.

Background

One aspect of understanding a specific cell type's contribution to organ function is to profile the cell's transcriptome. Previous methods of isolating messenger RNA (mRNA) began with extracting a specific organ, then profiling the entire pool of isolated RNA[1]. However, this method only yields data from the whole organ, and is quite biased by over-representation of transcripts from very prevalent cell types[2]. Furthermore, changing transcriptional profile under different conditions may simply represent a shift in cell population, for example from infiltrating leukocytes. Recently, researchers have recognized these issues and called for increased focus on the characterization of specific cell types[3]. As interest in the contribution of specific cell types to an organ's function increased, methods of isolating cells of interest by fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) were adapted to isolate cells based on their expression of characteristic surface markers[4-6]. It should be noted however, that in complex tissues, cells form various interactions with their neighbors and the extracellular matrix, complicating their extraction more than cell types in peripheral blood, for example[7]. Although many markers are well-characterized and seemingly restricted to only a specific cell type, some cells, such as perivascular stromal cells, are also identified by their location relative to certain organ structures. Armulik, et. al. describe that the best practice for identifying pericytes includes staining for validated markers, in addition to counter-staining for endothelial cells, and confirming morphology and localization within the tissue[8].

Laser capture microdissection (LCM) provided a method to isolate single cells based on their location in tissue, rather than expression of surface markers[9,10]. LCM can offer more specific information than whole-organ isolates about various cell types, based on their localization[11]. However, as LCM requires a very thin slice of tissue, (2-15um), a fixation step is typically required[2]. A similar method is the single-cell analysis of cells aspirated by patch pipette. This method allows the researcher to combine transcript and electrophysiological data from single cells[12-14]. However, the utility of this method is restricted to electrophysiologically active cells such as neurons and myocytes. Additionally, both LCM and patch pipette cell isolation generate very small amounts of RNA, as samples are collected from single cells.

Although first isolating a specific cell population, then lysing the cells and collecting RNA will uncover the abundance of specific mRNAs (and other RNA populations), abundance is only part of the picture. Ingolia, et. al., showed that the rate of translation of a particular transcript predicts protein abundance with much more accuracy than mRNA abundance alone[15]. However, translational rate correlates with protein abundance with an R^2 value of only 0.42, demonstrating that post-translational processing also plays a large role in protein production[15]. An estimate by Schwanhausser, et. al. agrees, indicating that only approximately 40% of variation in protein expression is due to mRNA abundance[16]. The relationship between mRNA and protein is not 1:1, and involves post-transcriptional modifications and varying efficiencies of translation[17]. In contrast, identification of specific transcripts that are actively

being translated provides more information about what proteins are actually being produced.

Methods of polysome enrichment by ultracentrifugation were adapted to isolate the population of RNA actively being translated by the ribosome to provide more complete information about protein production[18-20]. This technique offers interesting information about the relative efficiency of the translation of different transcripts, as efficiently translated transcripts are associated with "heavy" polysomes (>3 ribosomes), while poorly translated transcripts associate with lighter ribosomal layers in the sucrose gradient[21]. However, while this and the previous methods offer the ability to isolate specific cell populations, and even offer information about gene expression, they involve long processing times, and introduce experimental noise due to cell fixation, tissue dissociation, and incubation ex-vivo. With added time to disrupt tissue, label and fix cells, and other processing, mRNAs are degraded, and quickly transcribed messages already translated. Struhl, et. al. concluded based on a study of more than 21,000 yeast mRNA transcripts, that only 1% of mRNAs have a half-life of more than two hours, while the median half-life is only 32 minutes[22]. Based on the short-lived nature of mRNA, previous methods have limited ability to offer information about the entire transcriptome of the cell, and instead, select for only very stable transcripts.

Methods of affinity purification of translating ribosomes subvert these and other difficulties to isolate actively translated mRNA from a population of genetically defined cells. One method of gathering transcriptional data about a specific population of cells is by immunoprecipitating ribosomes and attached mRNA, using a genetically expressed affinity tag. Expression of this tag is driven by a cell-specific promoter, and its isolation provides a pool of actively translated mRNAs. This method does not require previous isolation of target cells or fixation of tissue, thus allowing for in situ profiling of various cell types with minimal disruption[7]. Various iterations of this method even include a fluorescent affinity tag for visualization of tagged ribosomes in tissue sections, and can also be isolated with commonly available antibodies against GFP, RFP, and the like[23,24]. The precipitation of translating ribosomes by this method has also been adapted to cells in culture, by transfection of a tagged ribosome expression construct[25]. Translating ribosome affinity purification (TRAP) has also been shown to be of use in isolating even very rare cell populations. The Junion group has been able to profile the transcriptome of only 100 muscle cells in the developing *Drosophila melanogaster* embryo[26]. This method allows the researcher to distinguish between the transcriptomes of different genetically defined cell populations, assess the response of specific cell populations to different biological states, and other applications.

TRAP methods are not without limitations, however. It is important to note that protein-coding mRNA associated with ribosomes constitutes a very small fraction of total cellular RNA. In an actively dividing cell, ribosomal RNA (rRNA) comprises about 80% of the cell's RNA, while protein-coding mRNA comprises only about 5%[27]. Not all available mRNA is translated, and there are many species of non-coding RNAs that might have important effects on protein output,

including microRNAs[28,29]. This consideration is especially crucial given the current understanding that translational repression is one of several ways that microRNAs can interfere with protein production[30]. It was first observed by Lee, et. al. that the gene *lin4* encodes a small RNA which has antisense complementarity to *lin14*, and decreases the abundance of LIN14 protein without decreasing abundance of its transcript[31]. Additionally, Pillai, et. al. and Olsen, et. al. showed that at least one method of translational repression by microRNAs occurs once the mRNA is already engaged with the ribosome, by preventing polyribosome formation[32,33].It is also important to note that this method does not provide information about the elongation step of translation. Transcripts on which the ribosome is "paused" or otherwise not completing translation will also be included in the pool of isolated RNA[30]. TRAP methods also rely on a genetic marker that is restricted to only the cell type of interest. As Kim, et. al., points out, this can be problematic for analyzing certain groups of cells, such as neuroblasts, which are identified by expression of several different markers[2].

Myriam Heiman first conceived of a method of isolating translating ribosomes by affinity purification in 2008, as a way to differentiate between different neuronal subtypes in the striatum[23]. To develop the method, the group screened different ribosomal protein/affinity tag fusion products for their subcellular localization and incorporation into the polysome complex. The fusion of EGFP and ribosomal protein L10a proved to be the most promising. To achieve expression of the EGFP-L10a fusion protein restricted to specific neuronal subtypes, the group utilized a bacterial artificial chromosome (BAC) system, placing EGFP-L10a expression under control of the D1 or D2 receptor. The group has also detailed their methods more thoroughly elsewhere[7].

Today, a variety of affinity tags, ribosomal proteins, and expression promoters have been validated using similar methods. This review will discuss various applications of these methods, points to consider when designing such an experiment, and tips gathered to ensure the success of cell-specific transcriptional analysis in complex tissues.

Current methods

Methods of affinity purification of translating ribosomes from complex tissues have expanded rapidly in the last ten or so years. A quick search of PubMed for "translating ribosome affinity purification" reveals 54 hits, encompassing a wide variety of research questions. This section will review applications of this technique and organ systems that it has been applied to, to date. Mouse lines that have been crossed for this technique are presented in Table 1.

Affinity purification of translating ribosomes typically is used to accomplish one of two things: 1) isolation and analysis of the pool of actively translated mRNAs or 2) profiling of the sites of ribosome occupancy along the mRNA transcript (ribosome footprinting). While both methods contribute information about the transcriptome, and specifically about actively translated transcripts, the latter also offers another layer of information, which more accurately predicts the

abundance of protein being made from a specific transcript, and can also be used to assess the rate of translation for a particular transcript[34].

A good example of isolation of actively translated mRNA for the purpose of analyzing the transcriptome without ribosome footprinting is a 2009 study by Elisenda Sanz, et. al.[35]. This group identified that ribosomal protein L22 (Rpl22), when fused with various affinity tags, incorporated into the polysome, and showed appropriate cellular localization[35]. They then synthesized a targeting vector containing the Rpl22 allele with a floxed native exon 4, followed by a duplicated exon 4 with three HA tags. This system allows expression of the native Rpl22 without the presence of Cre recombinase, but with Cre recombinase present, expression of an HA-tagged Rpl22. The group christened the mouse expressing this construct the "Ribotag" mouse. The Ribotag mouse was then crossed to several different mouse lines expressing Cre recombinase under the dopamine transporter (DAT, *Slc6a3*) promoter, to tag dopaminergic neurons, dopamine and cAMP-regulated phosphoprotein (DARPP-32, *Ppp1r1b*) promoter, to tag spiny neurons of the striatum, and anti-mullerian hormone (*Amh*) promoter, to tag Sertoli cells of the testis. In each of these models, HA-expression was observed by western blot and immunofluorescence, and pull-down of cell type-specific mRNA was successful. In the DAT-cre:Ribotag precipitates, a 15-fold enrichment for dopaminergic neuron-related transcripts was noted, while for the DARPP32-cre:Ribotag, a 5 to 6-fold increase in medium spiny neuron transcripts was noted, and similarly, a 5-fold enrichment in Sertoli cell transcripts was noted in the AMH-cre:Ribotag precipitate[35]. This study validated the Ribotag system as a method of immunoprecipitating translating mRNA in two neuronal and one testicular cell type. This group has been able to use the method to compare populations of testicular cells[36], and study different isoforms of the fragile X mental retardation-1 (FMR-1) transcript[37], both of which will be discussed further below.

A study by Nicholas Ingolia, also in 2009 provided a basis to apply ribosome footprinting to studies of translation[34]. The group designed a ribosome protection assay to pull down polysomes, and deep sequence the ~30nt regions of mRNA protected by the ribosome in *Saccharomyces cerevisiae*[34]. The polysomal fraction was prepared by sucrose ultracentrifugation, and the attached mRNA was treated with nuclease digestion. Ribosomes were then removed, leaving behind short spans of "protected" mRNA, which was converted to a cDNA library and deep-sequenced. This method lent such resolution to the position of the ribosome on the mRNA strand that the reading frame could be determined, as 75% of fragments began with the first nucleotide of a particular codon[34]. The authors were also able to measure the translational efficiency for 4648 different genes by comparing ribosome occupancy to mRNA abundance. While this method bypassed the complexities of isolating cells from complex tissues by first optimizing the method in a single-cell organism, this study sparked the idea that the ribosome can be frozen in place on the mRNA transcript and the sequence protected by the ribosome can provide information about protein abundance, translation speed, translational control. Interestingly, the Ingolia group has applied ribosome profiling in very novel ways

to show evidence of translation both anucleate cell populations[38], and outside of protein-coding regions[39].

There are currently two methods that govern the cell specificity of the affinity-tagged ribosomal protein construct. The first is a bacterial artificial chromosome (BAC) containing regulatory elements of a cell-type specific gene, and the second is a Cre-lox system, where Cre recombinase expression is driven by a cell-type specific promoter. An example of the first is the (BAC)-based system, as utilized by Heiman, et. al. as the basis of the TRAP method[23]. The BAC system of cell-specific expression grew out of the efforts of Gong, et. al. to create a library of mice expressing GFP under the control of BACs for every cell type of the central nervous system[40]. Under this method, a separate mouse must be engineered for each cell type of interest. A BAC is engineered to contain the regulatory sequences of a gene of interest, as well as a ribosomal protein sequence and an affinity tag. The transgenic animal is generated by pronuclear injection of the BAC into the mouse embryo. In cells where the gene of interest is expressed, the BAC will also be expressed, as the construct contains regulatory elements of the cell-specific gene. The second way to facilitate cell-specific expression of tagged ribosomal proteins requires the expression of Cre recombinase in the cell type of interest, driven by a promoter specific to that cell type. The Ribotag method is an example of this system[35]. The Cre driver mouse is crossed to a mouse engineered to express a floxed native exon in a ribosomal protein gene, followed by a second copy of that exon, containing an affinity tag. Upon Cre recombinase-mediated excision, the tagged copy of the floxed exon is expressed. It is important to note that this method makes use of the large number of Cre recombinase-expressing mouse lines available, rather than relying on the synthesis of a new mouse for every cell type, as in the original TRAP method. Another particular strength of the Ribotag method is that expression of the transgene is driven by a ubiquitous promoter, ribosomal protein L22 (Rpl22), rather than expression of a singular cell-specific marker. The TRAP method has recently been modified to, like the Ribotag method, utilize existing Cre recombinase mouse lines. That way, the sequence of the marker for the cell type of interest does not need to be included in the BAC, to mediate the transgene's expression. Instead, the BAC only contains the ribosomal protein sequence and the affinity tag, which are integrated into the stably expressed Rosa26 locus. This is important because although BACs can fit a large amount of genetic information, they have a low probability of success for very large genes (>200kb)[40]. The ribosomal protein sequence in the expression construct contains a preceding stop codon that is floxed out upon Cre recombinase expression in the cell type of interest[24], so that the tagged ribosomal protein is expressed only in cells expressing Cre. An advantage of both this and the Ribotag method is that expression of the transgene is driven by a ubiquitous promoter (Rosa26 or ribosomal protein), rather than a cell-type specific promoter, so that tagged protein will be expressed in high proportion in the cell[35]. These methods for engineering the genetic construct are summarized in figure 1.

Figure 1: Genetic constructs available for cell specific transcriptional analysis in complex tissues.
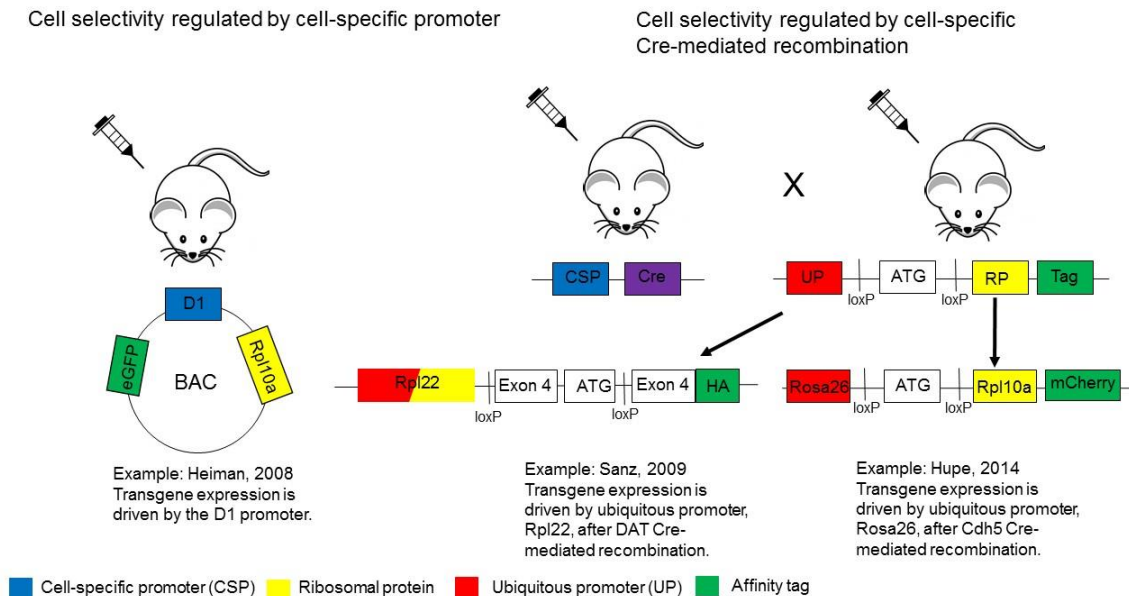
Actively translated mRNAs isolated by affinity purification can be either quantified by microarray or RNAsequencing. Microarray has for some time been the preferred method of transcript analysis, given the large number of genes that can be assayed at once and its relative availability. However, microarray relies on nucleic acid hybridization, detected by fluorescent probes. This can make detection of rare sequences problematic and similar sequences also have the potential issue of cross-hybridization[41]. RNAseq also has the added benefit of discriminating between different transcript isoforms for a particular gene, which may be of interest[42]. However, while RNAseq assays are becoming more approachable, microarray is still more available to many researchers. While the additional information provided by ribosome footprinting on transcriptional rate and efficiency for a particular transcript is alluring, this method also has its own drawbacks. For footprinting data to be accurate, one must be sure that the concentration of cycloheximide, a translation elongation inhibitor, is consistent throughout, or the data will be skewed, as cycloheximide preferentially inhibits elongation over initiation[33]. However, isolating mRNA and profiling by either RNAseq or microarray only requires an interaction with the RNA, not in a specific location[30]. In all, microarray is adequate for assaying the relative abundance of specific transcripts, but RNAseq is able to discriminate between different variants better, and when combined with ribosome footprinting, offers more information.

Studies in Brain

Many of the studies using translating ribosome affinity purification methods in specific cell types have been conducted in brain. With a variety of neuronal cell types and supportive cells, it is easy to see why assessing the contributions of different cell types in the brain to various biological processes would be important, a sampling of the applications of this method in brain follows.

In a study by Brackett, et. al., the group employed the Ribotag method to survey several different transcript isoforms of the fragile x mental retardation-1 (*FMR1*) gene[37]. The group identified 12 different transcript isoforms in adult mouse brain, and further wanted to investigate the dynamics by which specific isoforms are translated in neurons specifically, as they note that a large proportion of cells, 35-50%, in the brain are non-neuronal cell types. To assess translation of FMR1 transcript isoforms in neuronal cells specifically, excluding glial cells, tagging of neuronal ribosomes was achieved by crossing the Ribotag mouse with a mouse expressing Cre recombinase driven by the *Eno2* promoter. Using this method of isolating neuron-specific transcripts, the group noted about 3-fold enrichment for neuronal-specific transcript NeuN, and an approximately 0.5-fold depletion of glial transcript, GFAP. Isoforms 7 and 8 of the FMR1 transcript are most abundant in neurons from adult mouse brain, which is similar to the pattern of expression that the group noted from whole brain homogenate, suggesting that the expression of various isoforms of FMR1 is similar in neuronal and non-neuronal cell types[37].

Gonzalez et. al. applied the Ribotag method to a model of glioma in mouse brain[43]. In a very elegant application of this method, this group bred the Ribotag mouse with a mouse expressing floxed *Trp53* alleles. In the presence of cre recombinase and PDGF-B expressed by a retroviral vector, these mice develop glioma. The HA tag from the Ribotag is then restricted to cells which were originally transformed by the virus, allowing for isolation of tumor-specific mRNA. This alteration of the general technique subverts the fact that few markers reliably mark many transformed cells of a primary solid tumor. With the precipitated polysomes, the group opted to perform ribosome profiling by digesting the attached RNA and isolating the protected fragments. Translation rates were determined based on ribosome occupancy of the isolated transcripts, and pathways related to DNA replication, cell division, and ribosomal components were over-represented in the tumor-specific ribosome footprints, while synapse and cation channel activity pathways were associated with higher translation rates in normal brain[43].

Chandra, et. al. conducted a recent study into the same cell type as the Heintz group, the medium spiny neurons (MSN) of the striatum, as marked my the D1 and D2 promoters[44]. However, they utilized the Ribotag system, tagging Rpl22, and and sought to look at the differential effect of cocaine on these cell types. Expression of the transcription factor Egr3 is induced in striatum in response to acute cocaine stimulus, and the group wanted to assess which pool of MSNs contributed to this response. Immunoprecipitation of tagged ribosomes in the D1;Ribotag mouse resulted in enrichment of several D1-MSN transcripts including ~2.5-fold enrichment of Chrm4 and Tac1, ~5-fold enrichment of Drd1a, and ~13-fold enrichment of Pdyn. Immunoprecipitation from the D2;Ribotag mouse resulted in enrichment of D2-MSN associated transcripts, including ~2.5-fold enrichment for Drd2 and Gpr6, ~7-fold enrichment for Penk, and ~8-fold enrichment for Adora2a. Utilizing this method, the group found that D1-MSNs upregulate expression of Egr3 in response to cocaine exposure over one week, while Egr3 was reduced in D2-MSNs[44]. Overexpression of Egr3 in D1 MSNs

enhanced the conditioned place preference behavior, while overexpression in D2 MSNs reduced this behavior. Depletion of Egr3 in these cell types reversed these effects, suggesting that these cell types have opposing effects on the addictive behaviors accompanying cocaine use[44].

Studies in Vascular System

In another study to determine the contributing cell to a specific physiological effect, Everett, et. al. sought to explain whether endothelial cells or hepatocytes are the contributors of factor VIII to the coagulation cascade[45]. This group crossed the Ribotag mouse to a Tek/Tie2-cre mouse to express HA-tagged Rpl22 in endothelial cells. In liver lysate from Tek/Ribotag mice, the group saw 5 to 10-fold depletion of hepatocyte-associated transcripts albumin, prothrombin, coagulation factor V, coagulation factor VII, and coagulation factor X. The group also noted 15 to 22-fold enrichment of endothelial-associated transcripts cadherin 5, Tek tyrosine kinase, vascular cell adhesion molecule 1, and vonWillebrand factor. Enrichment of endothelial-associated transcripts in Tek/Ribotag mice was noted in several tissues: liver, kidney, brain, and heart[45]. In both liver and kidney lysates from Tek/Ribotag mice, transcripts immunoprecipitated with the HA tag showed enrichment for factor VIII, although this enrichment was greater in liver than in kidney (~10-fold and ~5-fold, respectively)[45].

A study by Santhosh and Huang elected to use the Tie2/Tek promoter to drive expression of the L10a-EGFP transgene in endothelial cells[46]. The authors compared the enrichment of endothelial-specific transcripts pulled down from mouse brain at embryonic day 15.5 (E 15.5) and postnatal day 0 (P0). At E15.5, PECAM and Tie2 were enriched 8 and 5-fold, respectively, while Wnt7a and Wnt7b were depleted 1.6 and 3-fold respectively. At P0, PECAM and Tie2 were enriched 36 and 12-fold respectively, while Wnt7a and Wnt7b were depleted 5 and 2-fold respectively[46].

Interestingly, this technique of translating ribosome affinity purification has also been applied to zebrafish. Fang, et. al. were able to express the Rpl10a-EGFP cassette driven by the cardiac myosin light chain 2a promoter in zebrafish cardiomyocytes[47]. The group noted PCR amplification of several cardiomyocyte-associated transcripts, hand2, cmlc2, vmhc, and anf in the RNA pulled down by immunoprecipitation in cmlc2/TRAP zebrafish heart ventricles. The total homogenate before immunoprecipitation contained these cardiomyocyte-associated transcripts in addition to non-cardiomyocyte-associated markers flk1, gata2a, and tcf21 however, there is no estimation of the degree of enrichment/depletion as the group didn't perform quantitative PCR. To profile cardiomyocyte gene expression changes after injury, the group employed a 20% apical resection to injure the heart. A large number of genes (138) were differentially expressed one day post-injury in the injured hearts versus uninjured controls. Notably, the group observed increases in the expression of several ion transporters and channels, supporting previous findings of changes in the electrical properties of cardiomyocytes during regeneration[47]. The group also

noted increases in expression of genes related to cell survival and proliferation[47].

<u>Studies in Reproductive System</u>

Spermatogenesis is supported by the Sertoli cell, which aids in the differentiation of spermatogonial stem cells into mature spermatozoa. Evans, et. al. chose to use the Ribotag method to assess transcriptional changes in this specific cell type in murine testis[48]. The group devised a novel technique of synchronizing the first wave of spermatogenesis in adolescent mice by introducing an inhibitor of retinaldehyde dehydrogenase, WIN18446, to arrest differentiation, followed by an injection of retinoic acid, to restart it. To enrich for mRNA from differentiating A, intermediate, type B, and a subset of undifferentiated A spermatogonia, the group crossed a *Stra8*-cre mouse to the Ribotag mouse. To mark Sertoli cells, the group utilized the *Amh*-cre mouse. Immunoprecipitation of RNA from Stra8/Ribotag mouse testis revealed 1-2 fold enrichment of germ cell transcripts including Stra8, Kit, Lin28a, and Ddx4, with 2-3 fold depletion of several Leydig and Sertoli cell-associated transcripts. The pool of enrichment for Sertoli cell associated transcripts, including Gpr56, Inhbb, Sox9, Gata1, and Fshr, with 1.5-2.5 fold depletion of Leydig and germ cell-associated transcripts. Microarray analysis of both pools of isolated RNA revealed 392 actively translated transcripts upregulated in germ cells and 194 in Sertoli cells[48]. In germ cells, these transcripts were clustered into meiosis, DNA binding, sexual reproduction, spermatogenesis, chromosome segregation, and DNA packaging and chromatin reorganization pathways. Pathways highlighted in Sertoli cell RNA however was more mysterious, and enriched for glycoprotein, cell adhesion, membrane, cell-substrate adhesion, cell junction, and adherens junction, all of which the authors suggest are involved in creation and maintenance of the blood-testis barrier[48]. The group also wanted to observe the timeline associated with DNA binding and chromatin regulation-associated transcripts in spermatogonia. They found a spike in expression of Asf1b at 6 days post-RA injection, in addition to several histone variants[48].

The McKnight group, which first published on the Ribotag system also applied this technique to the investigation of Sertoli cell-specific transcriptional response[36]. The group also utilized an Amh/Ribotag mouse as mentioned above to tag Sertoli cell ribosomes, and also used a Cyp17-iCre mouse crossed to the Ribotag mouse to generate Cyp17/Ribotag mice to tag Leydig cell ribosomes. The group noted 5-8 fold enrichment for Sertoli-cell associated transcripts, including Sox8, Sox9, Inhbb, and Gata1 in the Amh/Ribotag immunoprecipitate, with depletion of Leydig cell-associated transcripts Cyp11a1 and Prlr, as well as several germ line markers. Transcripts for Leydig-cell associated genes were enriched in the Cyp17/Ribotag immunoprecipitate. Transcripts including Prlr, Cyp11a1, Lchgr, and Star were all enriched 8-10 fold, while Sertoli cell transcripts, including Sox8 and Gata1 were roughly equivalent in the immunoprecipitate as compared to the total homogenate, and thus were not depleted in this model. The group used this method to identify novel Sertoli and Leydig cell-associated transcripts. Novel Sertoli cell-associated transcripts

included several receptors including the mannose receptor (Mrc1), vitamin D receptor (Vdr), as well as Calpain 6 (Capn6), among others. Gene ontology analysis showed that pathways related to regulation of cellular compartment movement, regulation of cell migration, sex determination, formate-tetrahydrofolate ligase activity, and phosphodiesterase 1 activity were enriched[36]. The group applied the same analysis to the Cyp17/Ribotag model and found several Leydig-specific markers including IL17 receptor, interferon receptor Ifnar2, low density lipoprotein receptor Ldlr, and G protein-coupled receptor Gpr128. Gene ontology analysis revealed that Leydig cell immunoprecipitates are enriched for transcripts related to steroidogenesis[36]. The group also treated Cyp17/Ribotag mice with a gonadotropin-relasing hormone (GnRH) antagonist followed by a single injection of leuteinizing hormone (LH) to observe the transcriptional response of Leydig cells to LH. There were 71 genes which had altered transcriptional status at one hour after LH administration. Two different transcription factors in particular increased dramatically in Leydig cells, Nr4a1 and Egr1[36].

Studies in Kidney

     Cell type-specific translational profiling experiments conducted in kidney have focused mainly on determining the role of different cell types in focal segmental glomerularsclerosis (FSGS) and acute kidney injury (AKI). Grgic, et. al. opted to utilize the TRAP method to observe transcriptional changes in podocytes using the Col1a1-cre to generate Col1a1-eGFP-L10a or Podo[TRAP] mice[49]. Importantly, the authors point out that there was no difference in proteinuria between transgenic mice and littermate controls, indicating normal function of podocytes in the Podo[TRAP] mice. The group noted approximately 200-fold enrichment for podocyte-associated transcripts nephrin, podocin, synaptopodin, and Wilms Tumor 1 (WT1). The group sought to identify transcripts that are highly associated with podocytes compared to the rest of the kidney cortex. By microarray, they noted that podocin, nestin, angiopoietin-like 2, protein tyrosine phosphatase receptor type O, nephrin, and synaptopodin all were very highly associated with podocytes[49]. To assess the podocyte's transcriptional profile during the course of FSGS, Podo[TRAP] mice were crossed to two different genetic mouse models of FSGS; the a-actinin 4 KO mouse and the a-actinin 4 point mutation K256E mouse, which harbors a point mutation associated with human disease. In the KO of a-actinin 4, the group noted an increase in translation of matrix-metalloproteinases 2 and 10, as well as connective tissue growth factor, NFkB inhibitor alpha, and the proinflammatory chemokines Cxcl1 and Cxcl10. Translationally increased transcripts in the a-actinin point mutation mouse were not similar to the profile seen in the KO mouse. In the a-actinin point mutation mouse, myotonic dystrophy protein kinase, actin-binding protein filament B, and cingulin were all translationally upregulated. Fittingly, pathways related to cytoskeleton reorganization were represented in the a-actinin point mutation mouse[49]. The contrasts between these two mouse models of FSGS point to two possible avenues by which podocyte malfunction might cause glomerular disease.

Liu, et. al. utilized the TRAP method to look at a variety of cell types in acute kidney injury (AKI)[50]. The eGFP-L10a construct was knocked in to the Rosa26 locus, but preceded by the SV40 polyA cassette, to be floxed out with introduction of cre recombinase, allowing transcription of the eGFP-L10a. The group generated Six2-L10a mice to mark nephrons, Foxd1-L10a mice to mark interstitial cells, Cdh5-L10a mice to mark endothelial cells, and Lyz2-L10a mice to mark myeloid cells. However, expression of these markers was not restricted to the presumed cell types, Foxd1-L10a marked interstitial cells as well as a subset of nephrons and a subpopulation of F4/80+ cells. Cdh5-L10a marked endothelium, but also a subset of F4/80+ cells. The Six2-L10a mouse marked not only nephrons, as expected, but also podocytes, which were expected to be marked by Foxd1. The Lyz2-L10a however seemed to be restricted to myeloid lineage cells. The group showed pulldown of one cell-specific transcript for each L10a mouse line, however, significant crossover was still observed. For example, a large amount of Foxd1 transcript is pulled down in the Six2-L10a mice, and Cdh5 is pulled down in the Lyz2 mice. The group performed a renal ischemia/reperfusion injury model of AKI by clamping the renal pedicles for 28 minutes. Both the Six2-L10a and the Foxd1-L10a immunoprecipitates contained many transcripts associated with renal tubule activity, including amino acid metabolism, small molecule biochemistry, lipid metabolism, and energy production[50]. Both the Lyz2-L10a and Cdh5-L10a immnoprecipitates had similar transcriptional signatures as well, with both showing enrichment for pathways related to inflammatory response and immune cell trafficking[50]. A transcriptional signature unique to Foxd1-L10a mice during IRI however, was activation of cell invasion and vasculogenesis programs. The ambitious attempt to identify roles for four different cell types in the context of AKI uncovered interesting findings, however, the lack of specificity of the eGFP-L10a strains for the cell types of interest makes attributing responses to specific cell types a challenge.

Studies in Liver
Very few studies using cell-specific ribosome profiling have been conducted in liver. Pack et. al. chose to profile translated mRNA from biliary epithelial cells in a zebrafish model to better understand how this cell type might contribute to cholestatic liver disease[51]. The group used the zebrafish keratin 18 promoter to drive expression of Rpl10a-eGFP in biliary epithelial cells of the zebrafish liver, and the LFA promoter to drive expression in hepatocytes. Immunoprecipitated mRNA from krt18-TRAP zebrafish liver showed enrichment for keratin 18 and cftr transcripts, which the authors identify as being specific for biliary cells[51]. mRNA from lfa-TRAP zebrafish liver showed enrichment for transferrin and lfabp transcripts, however both were also pulled down to a small degree in the krt18-TRAP immunoprecipitates as well[51]. The group demonstrated the use of this method in zebrafish liver and presumably will apply this to models of cholestatic liver disease in the future.

Table 1: Mouse lines generated for cell-type specific translational profiling.

Promoter	Cell type/Tissue	Expression construct	Reference
Eno2	Neuron/CNS	Cre, Ribotag	Brackett, 2013
Camk2a	Neuron/CNS	BAC, TRAP	Drane, 2014. Cook-Snyder, 2015
D1	Neuron/CNS	Cre, Ribotag	Heiman, 2014. Chandra, 2015
D2	Neuron/CNS	Cre, Ribotag and BAC, TRAP	Heiman, 2014 Chandra, 2015 Puighermanal, 2015
LepR	Neuron/CNS	Cre, TRAP	Allison, 2015
Emx1	Neuron, glia/CNS	Cre, TRAP	Hupe, 2014
Hcrt	Neuron/CNS	BAC, TRAP	Dalal, 2013
Chat	Neuron/CNS	BAC, TRAP	Doyle, 2008. Gorlich, 2013
Pcp2	Purkinje/CNS	BAC, TRAP	Doyle, 2008
Aldh1L1	Astrocyte/CNS	BAC, TRAP	Bellesi, 2015
CNP	Oligodendrocyte/CNS	BAC, TRAP	Bellesi, 2013
Sox2	Progenitor/CNS	Cre, TRAP	Hupe, 2014
Pax6a	Retinal ganglion cell/Brain	Cre, Ribotag	Shigeoka, 2016
Trp53	Malignancy/CNS	Cre, Ribotag	Gonzalez, 2014
Olig2	Malignancy/CNS	BAC, TRAP	Halliday, 2014
Tek/Tie2	Endothelial/Various	Cre, Ribotag, Cre, TRAP, and BAC, TRAP	Zhou, 2013. Everett, 2014 Santhosh, 2016
Cdh5	Endothelial/Various	Cre, TRAP	Liu, 2014. Hupe, 2014
Lyz2	Myeloid	Cre, TRAP	Liu, 2014
Foxd1	Interstitial/Various	Cre, TRAP	Liu, 2014
Stra8	Sprematogonia/Testis	Cre, Ribotag	Evans, 2014
Amh	Sertoli/Testis	Cre, Ribotag	Sanz, 2013. Evans, 2014. De Gendt, 2014
Cyp17	Leydig/Testis	Cre, Ribotag	Sanz, 2013
Col1A1	Podocyte/Kidney	Cre, TRAP	Grgic, 2014
Six2	Nephron/Kidney	Cre, TRAP	Liu, 2014
Albumin	Hepatocyte/Liver	Cre, Ribotag	Gao, 2015

Please note that Doyle, 2008 examines other mouse lines derived from the Gong, 2003 BAC generation project for CNS cell types.

Considerations for planning experiments

While the potential applications of this method are far-reaching and provide a wealth of information, transcriptome analysis is very complicated, and predictably, there are many potential pitfalls and considerations to make when planning an experiment of this kind. I will first describe the critical components of a typical translating ribosome affinity purification experiment, based on previously published protocols[7,35]. I will then detail several possible difficulties worth considering, and describe how we have overcome these.

The homogenization/lysis buffer used in a typical experiment is hypotonic and has an osmolarity of about 100mM. For reference, the osmolarity in human cells is about 150mM[52]. In addition to potassium chloride to establish osmolarity, all buffers must contain at least 12mM magnesium, which protects ribosomal integrity. The large ribosomal subunit contains 116 different magnesium ions[53], which help the ribosome function by binding RNA[53] and by supporting association between different helices of the large subunit[54]. Addition of several competitive RNase inhibitors, including heparin, helps to preserve RNA integrity. Heparin can increase the yield and prevent dissociation during polysome preparation, at a concentration of 0.75mg/mL [55]. However, according to Liu, et. al., removal of heparin by heparinase I treatment increases the yield of intact RNA from a rat lung model of ischemia/reperfusion injury [56]. Lungs were excised from the rat following heparinization with 1000IU/kg, which was sufficient to inhibit downstream reverse transcriptase, in the absence of heparinase treatment. The typical concentration of heparin for TRAP homogenization buffer is 1mg/mL, which is high enough to inhibit downstream assays. Cycloheximide is an inhibitor of eukaryotic translation, derived from *S.*

griseus[57,58]. Cycloheximide is typically included at a concentration of 100ug/mL to freeze the ribosome in place on the mRNA transcript. Addition of protease inhibitors helps to preserve the integrity of the ribosomal complex, as dissociation might prevent proper immunoprecipitation. These components are summarized in Table 2.

Table 2: Typical components of homogenization buffer for ribosome immunoprecipitation.

| Reagent	Concentration	Purpose
Potassium Chloride	100mM	Cell lysis
Magnesium Chloride	12mM	Ribosome protectant
RNasin	200U/mL	RNase inhibitor
SUPERasin	200U/mL	RNase inhibitor
Protease inhibitors	1x	Ribosome protectant
HEPES	20mM	Buffering agent
Cycloheximide	100ug/mL	Translation inhibitor
1,4-Dithiothreitol	1mM	Reducing agent
NP-40	1%	Membrane dissociation
Heparin	1mg/mL	RNase inhibitor
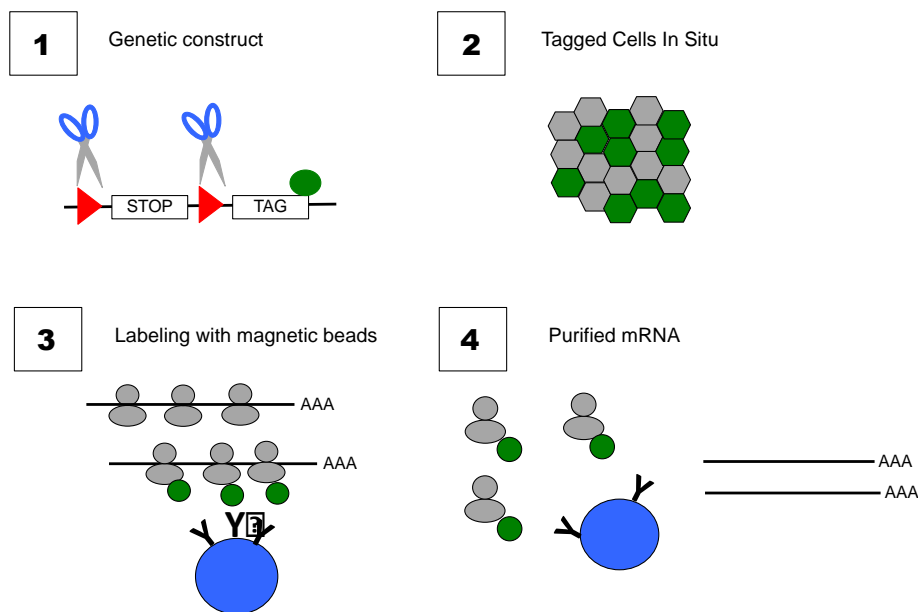
Figure 2: **Genetic construct (1):** Cre recombinase-mediated excision of stop codon preceding affinity tag allows expression in only the cell type of interest. **Tagged cells In Situ (2):** Ribosomes in targeted cells express tagged ribosomes (green), while ribosomes from non-targeted cells do not contain tag (gray). **Labeling with magnetic beads (3):** After tissue disruption, homogenate is incubated with antibody-labeled magnetic beads against the affinity tag. mRNA associated with tagged ribosomes (green) is pulled down, while mRNA associated with non-tagged ribosomes (gray) remains in solution. **Purified mRNA (4):** Washing beads removes unbound ribosome/mRNA complexes, and RNA is eluted. This results in purified RNA from only target cells.

Figure 2 outlines the key steps of this procedure, in the case of the BAC-Cre or Ribotag-Cre methods, where Cre recombinase expression is required. After buffer preparation, the tissue of interest is dissected from the animal, and placed into ice-cold buffer, then homogenized. It is extremely important to maintain RNase-free conditions, and keep conditions cold, to maximize the yield of the immunoprecipitation. The homogenate is then centrifuged to remove cell debris, and anti-affinity tag antibody is introduced to label the sample either directly (label sample with antibody, then add beads), or indirectly (label beads first with antibody, then introduce into sample). The beads are then washed with a high salt-containing solution, which increases the potassium chloride concentration to 300-500mM, to reduce non-specific binding. The immunoprecipitated RNA is then eluted by introducing a commercially available RNA stabilization reagent, such as Qiagen's buffer RLT, and RNA is purified.

RNA and ribosome stabilization

RNA integrity is of critical importance in these studies. We found a significant amount of RNA degradation in lung when using the concentration of RNase inhibitor recommended by Sanz et. al. Increasing RNase inhibitor concentration, combined with a ribosomal enrichment procedure described more fully below, increased the integrity of isolated RNA drastically. This may be due to differential rates of RNA degradation in different tissues. Lee, et. al. performed an intensive study on RNA degradation in different tissues post-mortem[59]. The authors point out that human specimens collected post-autopsy for research are subjected to sometimes long periods of time without proper storage, and a systematic analysis of the integrity of RNA from various human tissues had never before been completed. The authors sampled tissue from 80 different physical locations in the body from four donor bodies 3-5 hours postmortem, and rated RNA integrity on a scale of 1-5. An RNA integrity value of 5 represents intact RNA with strong 28S and 18S rRNA bands in a 2:1 ratio, while a value of 1 indicates extreme degradation with a smear of low molecular weight products. The authors found that CNS tissues had the most intact RNA, with a mean RNA integrity value of 3.8, while gastrointestinal tissues fared the worst, with a mean value of 1.9. Interestingly, respiratory tissue RNA was also significantly degraded, with values ranging from 1.0 to 2.6, depending on sampling location. Prostate tissue was not sampled, but according to a collaborator, RNA from this tissue also experiences significant degradation (David Morris, personal communication). It should be noted that tissue harvest and homogenization in TRAP and similar methods occurs immediately following animal expiration to minimize RNA degradation. However, the differences in degradation between tissues suggest that the specific local tissue environment affects RNA degradation to a large extent.

To preserve RNA integrity, we adapted a method of polysome purification by ultracentrifugation through a sucrose pad. This method is similar to a procedure previously described[60]. Tissue homogenate is layered over a 30% sucrose pad and ultracentrifuged at 41,000rpm using an SW41Ti rotor for three hours at four degrees Celsius. This results in a ribosomal pellet, which can be

resuspended in buffer and used for immunoprecipitation. RNases are separated from polysomes during this purification process, protecting the RNA. The ribosomal pellet is then resuspended in buffer without heparin, which can inhibit downstream applications, as noted above. Figure 3 demonstrates the improvement on RNA quality by ribosomal enrichment. Samples were taken at subsequent points during the ribosomal enrichment and IP, and analyzed for RNA integrity by the Agilent Bioanalyzer. Figure 4 demonstrates that the HA tag is present in the ribosomal pellet. Lungs from a WT mouse and a Foxd1 Cre +/-;Rpl22$^{HA/WT}$ mouse were homogenized, then ribosomes enriched by ultracentrifugation. Both the homogenate ("input") and ribosomal pellet were separated by gel electrophoresis, then probed for HA by western blot. The Ribotag cross results in a 23kD fusion protein, observed in both the input and pellet of the Foxd1 Cre +/-;Rpl22$^{HA/WT}$.
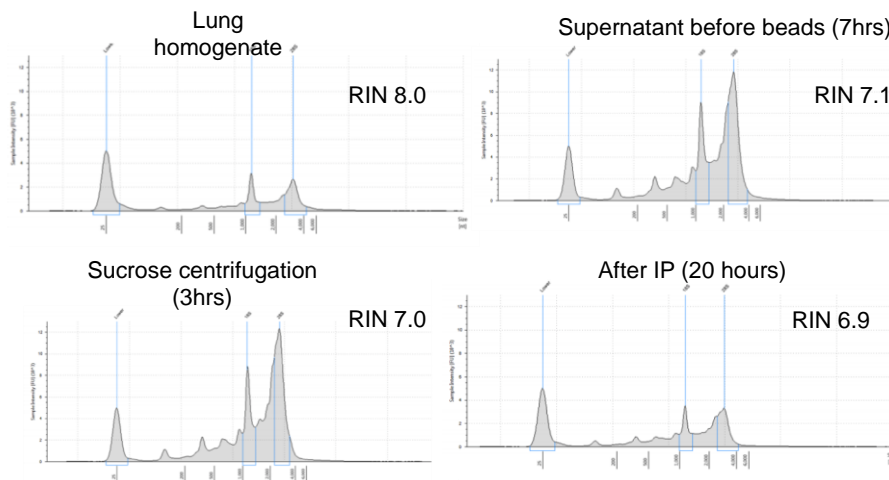

Figure 3: RNA stabilization by ribosomal enrichment. Lung homogenate was sampled for RNA integrity immediately after lysis, after ultracentrifugation, after antibody labeling, and after IP. RNA integrity assessed by Agilent Bioanalyzer, RNA integrity number (RIN) is on a 1-10 scale, with 1 being highly degraded, and 10 being highly intact.

Figure 4: HA tag present in homogenate and ribosomal pellet. WT and Rpl22 HA/WT;Foxd1cre +/- lung homogenates ("input) were sampled, then ribosomal pellets isolated by ultracentrifugation. Blot was probed for HA. The Rpl22-HA fusion protein results in a 23kD product.

Mouse considerations

The studies discussed above all rely on marking a genetically defined population of cells in order to pull down actively translated RNA from one specific pool of cells within a particular organ. Cre recombinase expression driven by a cell type-specific promoter is utilized in many of these studies, and a strength of this method is the vast array of Cre driver strains available. The Jackson Laboratory offers more than 300 different Cre recombinase-expressing mouse

strains, but many more have also been synthesized in various labs and are not part of the Jackson repository[61]. For many of these Cre lines, there are a great deal of data detailing the expression pattern of the driver gene. However, there are also many examples of genes that mark more than one cell type and this may be different under different biological conditions. For example, in the study by Liu, et. al., discussed above, there was significant overlap between interstitial cells, nephrons, and macrophages, between endothelial cells and macrophages, and between nephrons and podocytes[50]. Additionally, Sanz, et. al. mention that they observed off-target HA expression in non-Leydig cell types in their Cyp17/Ribotag mice[36].

Our recommendations for ensuring the specificity of affinity tag expression include best practices that are mostly adopted by our and other research groups. It is first of paramount importance to conduct co-localization studies in the desired tissue to be sure that the affinity tag is stably expressed in only the cell type of interest. In a mouse expressing the L10a-mCherry construct under the control Cdh5 (VEcadherin)-driven Cre recombinase (mCherry TRAP; Cdh5), we noted that mCherry colocalizes with CD31 (Pecam) expression (Figure 5). It is also important to assess whether the expression of the transgene varies in the biological state that one plans to interrogate. In our group, which studies Acute Lung Injury, it would be important to ensure that transgene expression does not change with injury, or the RNA isolated will not be comparable to that isolated in the absence of injury. In consideration of this, the choice of a Cre driver mouse which expresses Cre recombinase under the control of a gene expressed only during development, is wise. In addition to endothelium, our group is also interested in perivascular stromal cells (pericytes), which, in the lung arise from a group of progenitor cells that express Foxd1 during embryonic development[62]. To further improve the power of our data set, we have also crossed the Ribotag mouse to the PDGFRb cre mouse. PDGFRb is another marker of pericytes, and while it is not a lineage marker, it is more specific to pericytes than Foxd1. Comparing the RNA isolated using these two genetic models will give us greater certainty that our observations can be attributed to pericytes.
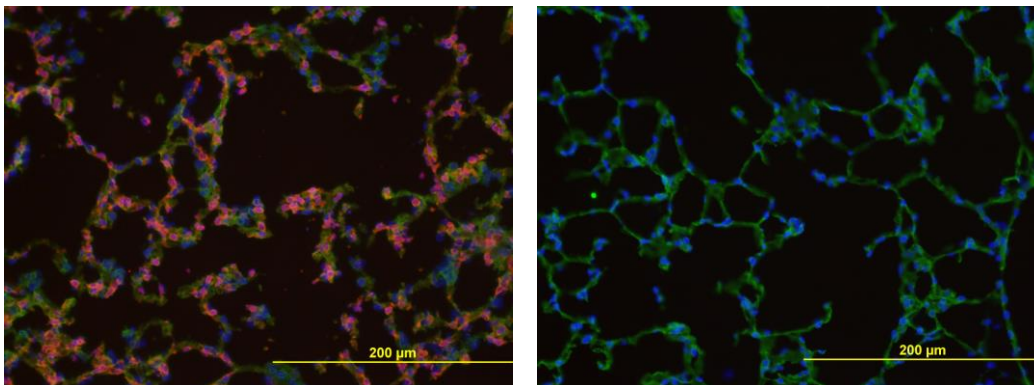

Figure 5: mCherry affinity tag overlaps with CD31. Lung sections were taken from Cdh5 cre;mCherry TRAP (left) and WT (right) mice and stained for mCherry (red) and CD31 (green).

While there are many considerations to make in the execution of these experiments, the information that cell-specific transcriptional profiling can yield is impressive. Translating ribosome affinity purification to isolate cell-specific mRNA overcomes issues of previous methods by restricting an affinity tag to a genetically defined cell population. Currently, there are a wide variety of mice available that give the researcher the ability to target many different cell types, through either BAC-driven or Cre recombinase-driven expression. TRAP also reduces processing time and isolates only actively translated, protein-coding mRNAs, as opposed to the entire pool of coding and non-coding RNAs. While this cannot offer insight into regulatory or non-coding RNAs, translated mRNAs have been shown to correlate well with protein expression. Our hope is that this information will aid other labs in optimizing this method for their studies.

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