Design considerations for analyzing protein translation regulation by condensates

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ABSTRACT
One proposed role for biomolecular condensates that contain RNA is translation regulation. In several specific contexts, translation has been shown to be modulated by the presence of a phase-separating protein and under conditions which promote phase separation, and likely many more await discovery. A powerful tool for determining the rules for condensate-dependent translation is the use of engineered RNA sequences, which can serve as reporters for translation efficiency. This Perspective will discuss design features to consider in engineering RNA reporters to determine the role of phase separation in translational regulation. Specifically, we will cover (i) how to engineer RNA sequence to recapitulate native protein/RNA interactions, (ii) the advantages and disadvantages for commonly used reporter RNA sequences, and (iii) important control experiments to distinguish between binding- and condensation-dependent translational repression. The goal of this review is to promote the design and application of faithful translation reporters to demonstrate a physiological role of biomolecular condensates in translation.

INTRODUCTION
Precise control of translation is essential for life. While all cells control translation location and timing, larger specialized cells have more elaborate RNA storage and transport requirements for normal functions owing to their size and shape (Ross Buchan 2014; Wang et al. 2021). Examples of these cells are neurons (Khalil et al. 2018; Pushpalatha and Besse 2019; Lan et al. 2021), muscle cells (Smith et al. 2020), oocytes (Cabral and Mowry 2020), and syncytial fungi (Zhang et al. 2015; Langdon et al. 2018). Consequences for disrupted spatially localized translation include neurodegenerative disease (Khalil et al. 2018; Elbaum-Garfinkle 2019) and infertility (Greenblatt and Spradling 2018; Riback et al. 2017); however, it is possible that condensation could lead to translation activation, although to our knowledge this has not yet been described. Mechanistically, condensates that lead to translational repression may exclude ribosomes, whereas translational activation could theoretically recruit ribosomes with or without essential cofactors. To further complicate matters, some RNA-binding proteins (for example, YBX1 and IMP1) can exist in either messenger ribonucleoprotein complexes (mRNP) (Mateu-Regué et al. 2019) with multiple proteins on a single mRNA or more macroscopic droplets (for YBX1; Liu et al. 2021) (for IMP1; Niewidok et al. 2018) depending on the co-condensing RNA. This suggests that binding can coexist in the same cytoplasm with and without phase separation and is at least partially dictated by features in the RNA component. Thus, additional work is needed to conclusively demonstrate a role of phase separation in translation regulation.
To convincingly demonstrate that phase separation, rather than RNA binding, is an essential emergent feature for localized and temporal control of translation, experiments can be undertaken to modify condensate components, and hopefully in turn, translation efficiency. Many of these experiments involve modifications of condensate protein components. These protein alterations include posttranslational modifications, such as phosphorylation or methylation (Tsang et al. 2019), or posttranslational modification mimetic mutations (Gerbich et al. 2020), altered protein sequence to mimic splice isoforms (Wiedner and Giudice 2021), disease mutations, or mutations designed with the intention of independently disrupting RNA-binding domains or intrinsic disordered regions. These experiments are invaluable but do not address the RNA component of the condensate entirely, leaving an incomplete understanding of how RNA is recruited to condensates, and whether recruitment leads to an alteration of translation efficiency.

Comparatively fewer experiments are done to demonstrate RNA sequence features which tune condensation and in turn translational regulation. There are two reasons why this may be the case: (i) multiple RNAs may be enriched in condensates and the necessary feature(s) for enrichment may be poorly understood; (ii) general hesitancy or unfamiliarity in the field to directly manipulate RNA sequence and structure features. Despite this, there are several shining examples of direct manipulation of RNA sequence, leading to altered condensate and cellular outcomes (Langdon et al. 2018; Cheng et al. 2021; Elguindy and Mendell 2021; Matheny et al. 2021a). We would suggest that similar undertakings with reporter RNA sequences could be used to more definitively distinguish a direct role for phase separation in translation regulation. This is because reporter RNA has the advantage over native target RNAs and protein products of having more sensitive and direct readouts using bioluminescence or fluorescence.

In this review, we will cover commonly used reporter RNA assay strategies and suggest how these reporters can be used to demonstrate condensate-dependent translational regulation. Thus, we will focus on how best to modify reporter RNA sequence to mimic features in condensate-enriched RNAs. The idea behind this is that by tuning condensation, one can more directly distinguish between the roles of RNA binding and condensation in translational regulation. Specifically, we will cover how to modify reporter sequences to mimic endogenous RNA targets, reporter RNA selection, and important control experiments. By modifying reporter mRNA sequence so that it (i) cannot be bound by condensate proteins, (ii) can only bind but not drive phase separation, or (iii) can lead to phase separation, it may be possible to demonstrate not only an understanding of the RNA requirements of the system but also generate evidence to evaluate if phase separation impacts translation regulation.

Part 1: How to modify reporters to mimic endogenous RNA targets?

In order to engineer a faithful reporter of phase-separation-dependent regulation of translation, it is essential to first establish the rules that govern RNA recruitment to the desired condensate (droplet) and then decide how to transfer these rules to reporter RNA sequences. Many phase-separating proteins contain canonical RNA-binding domains (KH, RGG, DEAD box, etc.) and noncanonical RNA-binding domains (i.e., charge, disorder). A discussion of how RNA-binding proteins recognize RNA sequences has been previously published (Corley et al. 2020). Design cues can also be taken from a list of enriched RNA targets (CLIP, etc.) and where possible, parse RNA targets from the condensed phase versus the diffuse phase by preventing phase separation of target protein via mutation (Halleger et al. 2021). Always helpful, although not essential, is determining if condensates are RNA dependent through condensate dissolution following RNase treatment (Fuller et al. 2020; Decker et al. 2021).

As a rule, it is also good to determine and take into account the position and the number of protein interaction motifs in an RNA of interest (5’ or 3’UTR vs. coding sequence, intron vs. exon, clustered vs. uniform distribution) (Singh et al. 2015). It is also advantageous to make as few primary sequence changes in reporter RNA as possible to be able to more easily compare to the original/native sequence. Finally, it is critical to compare mutant reporter RNA translation to the presence or absence of phase separation/condensates (the presence or absence of protein) (Cheng et al. 2021).

We first discuss mechanisms for how RNAs are recruited to condensates via protein recognition of RNA targets or RNA-intrinsic features. This information can inform how best to alter reporter RNA sequences so that the sequence recapitulates native protein/RNA interactions. The goal here is to describe examples of recruitment strategies from the literature for well-characterized condensates, which could be used as a design framework for newly discovered condensates.

Primary sequence motif

Some RNA-binding domains prefer a specific nucleotide sequence. To better recruit reporter RNA to the desired condensing protein, one can recode native reporter mRNA sequence through synonymous mutations to create additional binding sites, with the consideration of whenever possible preserving codon usage and RNA structure. Multiple examples of this exist for recoding Pumilio binding sites in its target, the long noncoding RNA, NORAD. Mutating Pumilio binding sites in NORAD/luciferase fusion RNA reduced the recruitment to stress granules (Matheny et al. 2021b) or blocked phase separation with Pumilio (Elguindy and Mendell 2021). Artificially tethering
luciferase RNA to increasing amounts of stress granule proteins had the opposite effect of enhanced recruitment to stress granules (Matheny et al. 2021b). In less well-studied targets, CLIP data can be used to identify consensus binding sites in primary sequences.

**Secondary structure-dependent motif**

Secondary structure is often an element of a protein-binding site in an RNA, and these may be part of a native system or engineered to create new interactions. Examples of commonly used engineered sites are MS2 (Tutucci et al. 2018) or PP7 stem-loops (Kim et al. 2019a). As an example of this form of native recruitment, RNAs with structurally complex 5′ UTRs preferentially condense with Ded1 following heat stress (Iserman et al. 2020a). To alter the reporter RNA to include a secondary structure motif, it is best to change the primary sequence either by appending a structured UTR or by changing the codon usage without changing the coding frequency. A caveat for interpreting the role of structure is that the RNA structure can also modulate protein expression through multiple mechanisms (Babendure et al. 2006; Mustoe et al. 2018; Mauger et al. 2019). We have recently demonstrated an example of this type of manipulation in which we enhanced the secondary structure of an N-protein target RNA fused to nano luciferase, demonstrating that it is better able to drive N-protein phase separation in a cell-free assay when compared to the native RNA structure (Roden et al. 2021).

**RNA length dependence**

Longer RNAs may have more opportunities to engage in transient interactions. It has been proposed for several phase-separating systems that longer RNA is more prone to enrichment stress granules (Khong and Parker 2018; Van Treeck et al. 2018), N-protein (Iserman et al. 2020b), FMR1 (Greenblatt and Spradling 2018), and P granules (Lee et al. 2020). Thus, to modulate the recruitment of RNA to a droplet, it may be possible to add an additional nonspecific or specific sequence to enhance droplet recruitment (Iserman et al. 2020b). Similarly, a control for the length should be using the shortest translation reporter available so as to have a non-phase-separating control RNA sequence. Notably, firefly luciferase can nonspecifically induce phase separation in vitro of multiple proteins (i.e., N-protein [Iserman et al. 2020b], FMR1 RGG [Kim et al. 2019b; Tsang et al. 2019]), and FXR1 isoform A and E (Smith et al. 2020), as it is among the longest reporter RNA sequence at ~1.7 kb and so can confound interpretation when there are insufficient negative controls that fail to phase separate.

**Translation efficiency/codon usage**

Poorly translated RNAs are preferentially recruited to several phase-separating systems (i.e., stress granules [Khong and Parker 2018] and P-bodies [Luo et al. 2021]). This is postulated to occur by reduced ribosome-mediated masking of RNA allowing for occupancy by RNA-binding proteins with the propensity to phase separate and increased trans RNA interaction. By modulating the codon usage of the reporter RNA sequence and in turn its translation efficiency, it may be possible to drive additional recruitment to droplets that rely on reduced translation as a recruitment mode such as stress granules, with the caveat that reporter protein levels will also be reduced and so appropriate controls are needed. Thus, by tuning the expression of a poorly translated RNA or RNA-binding protein to a particular cell type or cell state, it may be possible to leverage translational efficiency as recruitment for phase separation. For example, ribosome stalling and truncation due to poor codon usage in transposons are required for the recruitment to SGS3/RDR6 containing siRNA bodies (Kim et al. 2021).

**RNA modifications**

RNAs receive posttranscriptional modifications that can alter many properties of RNA. m6A is one of the most well-studied modifications and is known to regulate translation efficiency (Wang et al. 2015) and RNA stability. m6A RNA-binding proteins have been reported to undergo phase separation (Ries et al. 2019). RNA sequence can be modified to undergo modification by tuning the number and position of DRACH sites (the most favored modification sequence) in the RNA or by comparing ±METTL3 (the methyltransferase). Consideration should be paid to the positioning of the DRACH sites as in the native context, and these are often enriched around the stop codon (Meyer et al. 2012). An example of the manipulation of the DRACH site for translational regulation has been demonstrated for MYC/YTHDC1, where mutating m6A modification sites in the Myc coding sequence no longer reduce Renilla luciferase translation of a fusion RNA when YTHDC1 is knocked down. Furthermore, YTHDC1 protein needs to undergo phase separation in order to repress modified target RNA (Cheng et al. 2021).

**RNA:RNA interactions**

Target RNA may be recruited to droplets by trans RNA/RNA interactions rather than specific interactions with an RNA-binding domain (Van Treeck and Parker 2018; Van Treeck et al. 2018). These interactions can be transient and nonspecific in the case of stress granules (Van Treeck and Parker 2018) and TIS granules (Ma et al. 2021) and homotypic self-assembly of RNA in germ granules (Trcek et al. 2020). To nonspecifically promote RNA:RNA interaction in a reporter, lengthen the RNA sequence and reduce the structure and/or translation efficiency. It is also possible to promote specific RNA:RNA interactions by fusing a reporter RNA to a granule target RNA sequence, which forms dimers or higher multimers. For example, fusing GFP to
oskar 3′UTR allows for the recruitment of GFP to granules owing to the ability of oskar to dimerize in vivo. Reducing propensity of the UTR to dimerize reduces recruitment (Jambor et al. 2011). Additionally, the RNA structure can be used to mask sites of potential RNA:RNA interaction keeping droplet RNA contents immiscible. Such experiments have been undertaken for the Whi3/CLN3 Whi3/BN11 system where the RNA structure in CLN3 is sufficient to sequester CLN3 droplets from BNI1 droplets in a common cytoplasm (Langdon et al. 2018).

Thus, there are multiple described strategies employed by granules for enriching target RNAs. By altering reporter RNA sequence to resemble features of RNAs enriched in a specific native condensate, it may be possible to promote translational regulation by a particular phase-separating protein and begin to demonstrate a condensation-mediated regulation of translation; particularly if sequence modifications can be identified that parse RNA-binding and phase separation.

Part 2: Choosing the best translation reporter RNA sequence for your experiment

The method of measuring a reporter RNA’s translation depends on available equipment and sensitivity/specificity requirements. Particularly for in-cell experiments, if possible, one should include a second independent reporter RNA sequence that does not phase separate as a negative control. Ideally, this sequence is in the same plasmid and under the control of the same promoter or a bidirectional promoter to control for transcription levels (Van Etten et al. 2013). It is important to also demonstrate that the second reporter RNA is not or minimally expected to be regulated by the phase-separating protein/condensates of interest to control for basal translation level (firefly/renilla, GFP/ RFP). Inclusion of a translation inert reporter is less of a concern for cell-free experiments as reporter RNA levels can be more precisely controlled.

Three possible experimental strategies for translation reporters are as follows.

Light production

There are three commonly used and commercially available light-generating enzymes: Renilla, Firefly, and Nano luciferase (England et al. 2016). In-cell luminescence assays involve transduction (transient or stable) with DNA/RNA to produce the enzyme in question followed by the introduction of the enzyme substrate to produce light. Light production is correlated with protein levels and can be used to estimate the translated amount. Many of these assays require cell lysis, but some variations can be performed on live cells and in whole animals (Otto-Duessel et al. 2006; Zinn et al. 2012; Gaspar et al. 2021). An additional advantage to the light production is that translation can also be performed cell free (utilizing purified ribosomes and amino acids) (Fig. 1; Kim et al. 2019b; Tsang et al. 2019; Roden et al. 2021). Thus, the same engineered sequence can be measured for translation in and out of cells to more thoroughly demonstrate that translation control is specific to the minimal supplied components. Table 1 discusses some advantages to guide the selection of any one of these luminescent RNA sequences.

Fluorescent protein production

These assays involve transduction (transient or stable) with DNA/RNA to produce fluorescent protein. For example, GFP/RFP/BFP (one color for reporter RNA, control RNA, and phase-separating fusion protein) is a color combination with minimal spectral overlap. Measurement of fluorescent protein production can be of comparable price and time investment to those assays, which involve light production, depending on the detection method. Protein production can be measured by microscopy, flow cytometry, or western blot to examine bulk population levels. The main advantage of the use of a fluorescent protein is that it allows for the evaluation of cell–cell heterogeneity and correlation of condensate properties with output at the level of a single cell. It may also be possible to screen to determine the mode of regulation by cell sorting if the sequence can be appended to either 5′ or 3′ untranslated regions (Fig. 2). To our knowledge, a screen has not yet been demonstrated, but Ford et al. were able to develop a fluorescent reporter for phase-separation-independent CPEB3 translational repression by fusing the GluA2 3′UTR to DsRed, suggesting that the concept is sound (Ford et al. 2019).

Nascent polypeptide and RNA detection in situ

The simultaneous labeling of RNA with nascently translating peptides is a useful tool for examining translation in and out of a droplet in a live cell (Fig. 3). To our knowledge, three variations of this have been described, which differ most significantly in the method of detection for nascent polypeptides. (i) SINAPs that utilize a GFP conjugated to an antibody against GCN4 (Wu et al. 2016), such as a SunTag (Mateju et al. 2020). (ii) A genetically encoded camelid antibody (Moon tag, see below in Fig. 3) fused to a fluorescent or Halo tag (Boersma et al. 2019). (iii) Another approach (Morisaki et al. 2016) involves utilizing multiple nascently translated FLAG tags recognized by a fluorescent antibody fragment. By using this method in living cells, Moon et al. were able to show that mRNAs ceased to be translated before entering the SG and resume translation following stress granule disassembly (Moon et al. 2019). Contradictory results for SG translation were observed by Mateju using the SunTag system for a subset of RNAs (Mateju et al. 2020).
There are advantages and disadvantages to each reporter RNA system. Whereas the most used RNA is firefly luciferase, this RNA may not faithfully recapitulate some native condensate RNA features. Firefly luciferase nonspecifically induces phase separation of multiple proteins in cell-free assays and cannot be used for single-cell and single condensates in cell translation assays. Thus, the native firefly RNA sequence is a useful first-pass RNA reporter for translation, particularly for comparison of alterations to protein sequence, but subsequent experiments are needed to more conclusively demonstrate translation regulation on more physiological sequences.

### Part 3: Control experiments: confirmation of protein binding to reporter RNA and phase-separation-dependent modulation of translation

A final advantage of engineering an RNA reporter for translation is the demonstration of an understanding of the rules which can govern phase separation for a particular system. Thus, the engineered reporter RNA can be used to demonstrate that altered RNA sequence is sufficient to (i) be preferentially bound by a phase-separating protein, (ii) induce phase separation cell free and in cells, and (iii) is localized to the desired biomolecular

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**TABLE 1.** Comparison of luminescent RNA reporter sequences

| RNA     | Length | RNA for sale? | Substrate (s)                | Relative signal (firefly) | Whole organism                                      | Example proteins for unmodified (to enhance binding) RNA sequence |
|---------|--------|---------------|------------------------------|---------------------------|------------------------------------------------------|---------------------------------------------------------------|
| Firefly | ~1.7 kb| https://www.amsbio.com/firefly-luciferase-fluc-mrna-7001-203-1mg | d-luciferin, N/A            | N/A                       | Ye (Zinn et al. 2012)                                 | FMR1, FXR1, SARS-Cov-2 nucleocapsid                           |
| Renilla | ~0.9 kb| https://www.trilinkbiotech.com/cleancap-renilla-luc-mrna-5mou.html | Coelenterazine, EnduRen, ViviRen (Otto-Duessel et al. 2006) | Coelenterazine (17,000), Furimazine (15) (Hall et al. 2012) | Ye (Otto-Duessel et al. 2006)                                 |                                                                 |
| Nano    | ~0.5 kb| No            | Furimazine, Hydrofurimazine, | Fluorofurimazine, hikarazine-001, hikarazine-003, and hikarazine-097\* (Gaspar et al. 2021) | Coelenterazine (81,000), Furimazine (2,500,000) (Hall et al. 2012) | Yes (Gaspar et al. 2021)                                      |
FIGURE 2. In vivo reporter/screen design. A DOX-inducible phase-separating protein is fused to GFP, and a target RNA located in either the 5′- or 3′ UTR is fused to mCherry. Many variations of the target RNA can be cloned. Cells are sorted for intermediate mCherry signal to control for non-phase-separating translational repressive effects. Following induction of the GFP tagged phase-separating protein, reduction or increase in mCherry signal may be caused by phase-separation-mediated translation repression. Sorting for cells followed by sequencing can reveal enriched or depleted features.

FIGURE 3. Moon Tag for in vivo droplet translation visualization. RNA is labeled with PP7 stem-loops, and translated protein is recognized by a nanobody fused to a fluorescent protein. The advantage of this assay is that it allows for observation of nascent translation in- or outside of a phase-separated condensate in a living cell. This represents the lowest throughput but highest possible standard for condensate-dependent translational regulation.
condensate and no other condensates, if the following control experiments are employed.

**EMSA or similar binding or turbidity assay ± engineered mutations**

This control demonstrates increased protein affinity for the altered RNA reporter. Elguindy and Mendell (2021) were able to demonstrate that four or more Pumilio binding sites promoted Pumilio phase separation at lower concentrations of RNA.

**Differential phase-separation phenotypes: cell free**

In the ideal scenario, that is, cell free, the native (reporter RNA that is unmodified to have specific binding to protein of interest) reporter RNA sequence should not induce phase separation, making it the best negative control. Thus, alterations of RNA sequence should favor phase separation. This can be demonstrated with a phase diagram in cell-free conditions. Additionally, in the case of luciferase, cell-free translation assays should be under phase-separation promoting and inhibitory conditions. In our recent article, we utilized this approach to demonstrate that the addition of dsRNA enhances N-protein translation repression (Roden et al. 2021).

**RNA F.I.S.H. in vivo to confirm localization to condensates**

It is important to assess localization and enrichment of engineered RNA to the desired condensates. As an example of this, Elguindy et al. were able to demonstrate that four or more Pumilio binding sites promoted colocalization with Pumilio puncta in cells (Elguindy and Mendell 2021). Ideally, the same probes should be used for all tested sequences, thus careful design should be used to not disrupt the probe site when optimizing the sequence for phase separation. Assaying the compaction state by designing FISH probes for the 5’ and 3’ end may also be useful, as translating RNAs are more extended in the cytoplasm than those that localize to stress granules (Adivarahan et al. 2018; Khong and Parker 2018).

**QPCR/RNA-seq**

It is important to assess if reporter RNA levels are comparable with control RNA over the course of the experiment for in-cell experiments to demonstrate that mutated RNA sequences are at comparable levels over the course of the experiment. Care should be taken to design amplicons with identical sequence in the case of qPCR. As an example of this, Elguindy and Mendell (2021) were able to demonstrate that only RNA with four or more Pumilio binding sites was able to rescue NORAD activity and Pumilio phase separation in NORAD depleted cells while having similar to slightly lower RNA copy numbers per cell.

**CONCLUSION**

Engineered reporter RNAs represent a powerful and underutilized system for the demonstration of phase-separation-dependent translation regulation. Biomolecular condensate-mediated translational control has long been postulated and is beginning to be experimentally demonstrated. By altering the sequence of reporter RNA to modulate phase behavior, it is possible to more efficiently and specifically recruit reporter RNA to a desired target condensate and more precisely demonstrate a role for phase separation in translational regulation. While thus far, most described examples have indicated that condensation leads to translational repression, it is possible that condensation could also lead to translational activation, which could be directly visualized in live cells by simultaneously detecting nascent protein production, RNA, and condensate protein (see Fig. 2).

An essential unfulfilled need in the field is the development of new, or refinement of, existing assays to convincingly parse protein binding and phase separation. Additionally, in this review, we suggest two possible strategies for gaining additional information on cell–cell heterogeneity of translation control by condensation using fluorescent protein reporters, and we suggest possible parameters for a screen to unbiasedly identify RNA features that promote regulation by a phase-separating protein.

It is our hope that this review can serve as inspiration to overcome hesitancy to manipulating RNA sequence and structure not only to demonstrate condensate-mediated translation regulation but also other suggested physiological roles of condensates, which are more difficult to experimentally measure. We think there is a lot of unexplored and exciting biology for the roles of RNA sequence and structure in phase separation, and we look forward to reading your work!

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