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Isolation of mammalian stress granule cores for RNA-Seq analysis

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ABSTRACT

Stress granules are dynamic, conserved non-translating RNA-protein assemblies that form during cellular stress and are related to pathological aggregates in many neurodegenerative diseases. Mammalian stress granules contain stable structures, referred to as "cores" that can be biochemically purified. Herein, we describe a step-by-step guide on how to isolate RNA from stress granule cores for RNA-Seq analysis. We also describe a methodology for validating the RNA-Seq results by single molecule FISH and how to quantify the single molecule FISH results. These protocols provide a starting point for describing the RNA content of stress granules and may assist in the discovery of the assembly mechanisms and functions of stress granules in a variety of biological contexts.

1. Description of theoretical basis and framework for the technique

Stress Granules (SGs) are conserved non-translating RNA-protein (RNP) assemblies that form during cellular stress when translation initiation becomes limiting [1]. SGs are of biological interest for their roles in the stress response, as well as affecting tumor progression, neurodegenerative diseases, and viral infections [1–10]. Despite their biological importance, how SGs assemble and affect cellular responses to stress are not well understood. These questions have been challenging to address because little information is available on the RNA content of SGs. Importantly, we have recently developed methods to purify, sequence, and validate the RNA content of SGs in yeast and mammalian cells [23].

Our purification approach is based on the observation that SGs are biphasic RNP assemblies comprised of a dynamic outer "shell" and more stable internal "cores", which can be biochemically purified. We took advantage of this to characterize the proteome and transcriptome of SG cores. About half of the proteins that are localized in SGs have RNA-binding activity [11]. In addition, many of the proteins found in SGs have intrinsically disordered regions (IDR) which can facilitate protein-protein interactions and contribute to granule formation [12–18]. With respect to RNA, we discovered SG cores are mostly composed of mRNAs. However, the percent of copies of a given mRNA that localize to SG cores can vary from <1% to >95%. In addition, we discovered poor translation and transcript length are the predominant metrics for mRNA accumulation in SG cores [23].

Despite the fact that mammalian SGs have a dense core and a less concentrated shell region, two observations suggest we are identifying most of the RNA found in SGs when purifying SG cores [23]. First, there is a strong correlation between our RNA-Seq results and the partitioning of specific mRNAs in SGs as determined by single molecule FISH. Second, our estimate of the total number of mRNA molecules in SG cores as determined by RNA-Seq is similar to the estimated abundance of mRNA molecules in SGs as determined by oligo(dT) FISH. These results suggest we are capturing most of the SG RNAs with our protocol.

Previously, we described how to purify yeast and mammalian SG cores [11] ([24], Methods). In this methods paper, we describe how we adapted that protocol for the isolation and analysis of mammalian SG core RNAs [23]. The key steps for SG core isolation are sequential centrifugation to enrich for SG cores and purification of cores by immunoprecipitation. We also describe approaches to validate and quantify the RNA-Seq results by single molecule FISH (smFISH) using Stellaris RNA FISH and Bitplane Imaris imaging software respectively.

2. Transcriptome analysis of RNA in SG cores

2.1. Isolation of RNAs from mammalian SG cores

The key steps in isolating SG cores are sequential centrifugation to enrich for SG cores and purification of cores by immunoprecipitation using antibodies to either an accessible SG component or antibodies to a tagged SG component.

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1. Seed U-2 OS cells expressing G3BP1-GFP at ~40% confluent in three 500 cm^2 Square Bioassay Dishes (07-200-599, Fisher Scientific), and grow overnight. We recommend isolating SG cores from cells that are ~80% confluent. This protocol is sufficient for isolating enough RNA for one RNA-Seq experiment.
2. Exchange media 60′ before induction of stress with fresh media. (Make sure media is at 37 °C to ensure robust SG formation. Adding media colder than 37 °C can inhibit SG formation [19]).
3. Apply preferred method for inducing SGs (For a list of commonly used methods to induce SGs, see [20]). We have described the SG transcriptome by adding a stock solution of arsenite to media with a final concentration of 500 μM $NaAsO_2$ for 60′ incubation [23].
4. Aspirate media. Wash cells with 30 mL of media pre-warmed to 37 °C and add 15 mL of pre-warmed media to each dish.
5. Scrape cells and collect into a 50 mL conical tube.
6. Pellet cells at 1500×g for 3′ at room temperature. (We recommend steps 4–6 should be performed in less than 10′ to avoid formation of SG cores in unstressed control cells).
7. Aspirate media and flash freeze pellet in liquid N_2. (Stopping point: Cell pellets can be stored at −80 °C).
8. Thaw pellet on ice for 5′.
9. Re-suspend in 1 mL SG lysis buffer.
10. Lyse cells by passing resuspended cells through a 25G 5/8 needle seven times while on ice (See flowchart in Fig. 1 for steps 9–28).
11. After lysis, transfer lysate to a microcentrifuge tube, and spin at 1000×g for 5′ at 4 °C to remove cell debris. Discard pellet and transfer supernatant to a new microcentrifuge tube.
12. 50 μL of the lysate should be transferred to a new microcentrifuge tube to isolate total RNA. Trizol LTS manufacturer's protocol can be used to isolate RNA.
13. Spin the remaining lysate at 18,000×g for 20′ at 4 °C to enrich for SG cores. Discard supernatant.
14. Re-suspend pellet in 1 mL SG lysis buffer, and repeat step 13–14.
15. Re-suspend pellet in 300 μL SG lysis buffer.
16. Spin at 850×g for 2′ at 4 °C. Transfer supernatant to a new microcentrifuge tube. The supernatant represents the SG core enriched fraction.
17. Pre-clear SG enriched fraction by adding 60 μL of lysis-buffer equilibrated DEPC-treated Dynabeads. (For protocol on preparing dynabeads, see discussion of materials). Rotate on a nutator at 4 °C for 30′.
18. Remove Dynabeads using a magnet and transfer supernatant to a new microcentrifuge tube.
19. Repeat steps 17–18.
20. Add 20 μL of α-GFP antibody. Rotate on a nutator at 4 °C for 60′. This step can be done overnight.
21. Spin at 18,000×g for 20′ at 4 °C. Discard supernatant to remove unbound antibody.
22. Re-suspend pellet in 500 μL of SG lysis buffer and add 60 μL of equilibrated DEPC-treated Dynabeads. Rotate on a nutator for 180′ at 4 °C.
23. Magnet-separate Dynabeads and remove supernatant. Add 1 ml of Wash Buffer 1 and rotate on a nutator for 5′ at 4 °C. Repeat twice for a total of 3 washes.
24. Remove supernatant and add 1 ml of Wash buffer 2 and rotate on a nutator for 5′ at 4 °C.
25. Remove supernatant and add 1 ml of Wash buffer 3 and rotate on a nutator for 2′ at 4 °C.

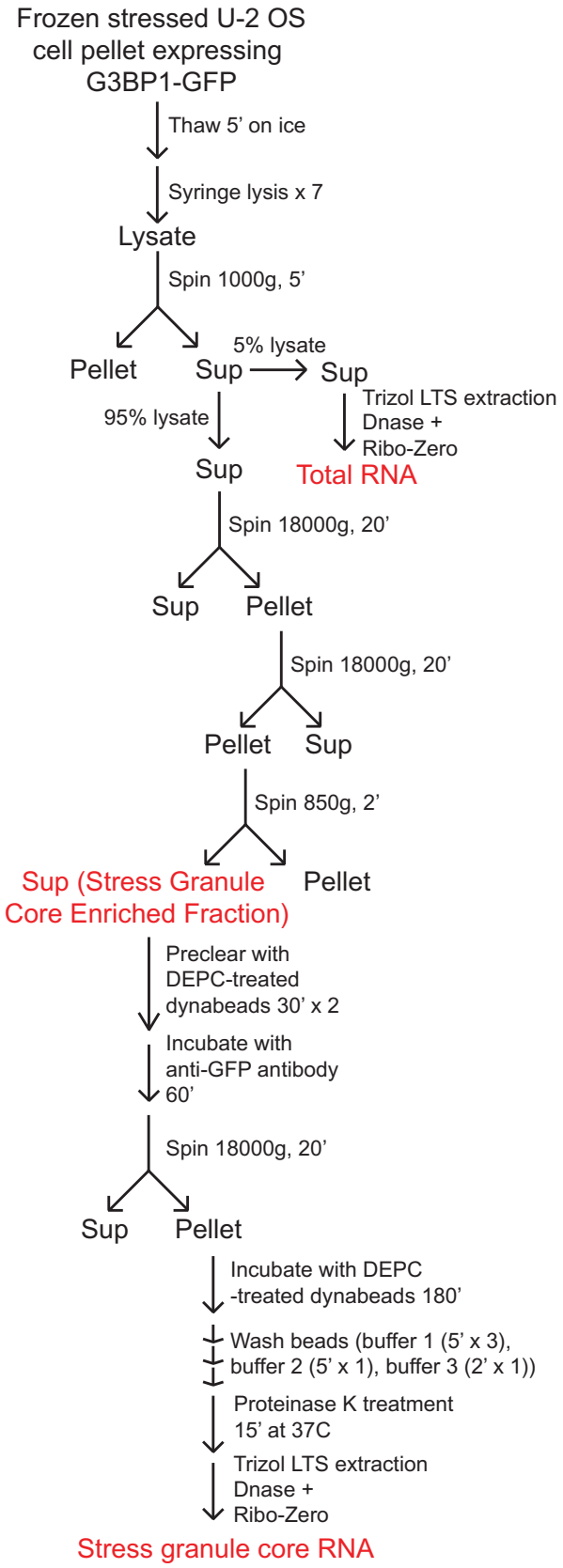

Fig. 1. Isolation of mammalian SG core RNA for RNA-Seq analysis. Schematic illustrating protocol for RNA isolation from SG cores. See Section 2 for a step-by-step guide.