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Isolation of mammalian SG 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.

Keywords

Stress granule; RNP granule; Purification; RNA-Seq; single molecule FISH

1.1 Description of theoretical basis and framework for the technique

SGs (SG) 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 (Khong et al., submitted).

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

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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 (Khong et al., submitted).

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 (Khong et al., submitted). 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 stress granule RNAs with our protocol.

In this paper, we provide a detailed protocol for the isolation and analysis of mammalian SG core RNAs. 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 using Stellaris RNA FISH and Bitplane Imaris imaging software respectively.

2.1 Transcriptome analysis of RNA in SG cores

2.1.1 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.

1. Seed U-2 OS cells expressing G3BP1-GFP at ~40% confluent in three 500cm² 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 NaAsO₂ for 60' incubation (Khong et al., submitted).
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 $1,500 \times g$ 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 N₂. (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 time while on ice (See flowchart in Figure 1 for steps 9–28).
11. After lysis, transfer lysate to a microcentrifuge tube, and spin at $1,000 \times g$ 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 $18,000 \times g$ 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 $850 \times g$ 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 equipment.) 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 16–17.
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 $18,000 \times g$ for 20' at 4°C . Discards 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 1ml of Wash Buffer 1 and rotate on a nutator for 5' at 4°C . Repeat twice.
24. Remove supernatant and add 1ml of Wash buffer 2 and rotate on a nutator for 5' at 4°C .

25. Remove supernatant and add 1ml of Wash buffer 3 and rotate on a nutator for 2' at 4°C.
26. Remove supernatant and re-suspend dynabeads in 250 µL 1X Proteinase K buffer at incubate at 37°C for 15'. (Protease digesting SG core proteins helps to get better RNA yields).
27. Remove supernatant and add 750 µL of Trizol LTS and proceed with RNA extraction following manufacturer's protocol. (We recommend adding 0.2 µg/µL glycogen during isopropanol precipitation to improve RNA yield and for visualizing pellet)
28. Re-suspend Total RNA (see step #12) and SG core RNA in 50 µL and 20 µL of RNase-free water respectively. (Stopping point: RNA can be stored at -80°C)

2.1.2 Prepare RNA for cDNA library construction and RNA-Sequencing

The key steps in preparing isolated RNA for library construction are (1) DNase-treating the RNA, (2) inspecting RNA quality, and (3) depleting rRNA from the RNA samples.

1. Dilute 1 µL of Total RNA in 16 µL RNase-free water.
2. Take 17 µL of SG core RNA and 17 µL of diluted Total RNA and add 2 µL of 10X DNase I Buffer and 1 µL of rDNase I to each.
3. Incubate at 37°C for 30'.
4. Add 2.2 µL of DNase Inactivation Reagent. Mix well.
5. Incubate for 2' at room temperature. Mix occasionally.
6. Centrifuge at $10,000 \times g$ for 1.5'. Transfer RNA to a fresh tube. Avoid disturbing the pellet containing DNase Inactivation Reagent. (Stopping point: DNase-treated RNA can be stored at -80°C)
7. Inspect the quality of RNA using high sensitivity RNA ScreenTape (Agilent, 5067-5579) on Agilent TapeStation 2200 instrument following manufacturer's protocol. (We aim for a RIN score of more than 8).
8. Ribosome deplete the RNA using Ribo-Zero rRNA removal kit following manufacturer's protocol (Illumina, MRZH11124). (Stopping point: Ribosome depleted RNA can be stored at -80°C)
9. Confirm ribosomal RNAs are depleted by inspecting the RNA using high sensitivity RNA ScreenTape (Agilent, 5067-5579) on Agilent TapeStation 2200 instrument following manufacturer's protocol. Measure amount of ribosome depleted RNA using Qubit.
10. Prepare cDNA libraries following manufacturer's protocol from 10 ng ribosome depleted total and SG-core RNA using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, E7420S).

11. Examine the quality and amount of cDNA libraries using high sensitivity D1000 ScreenTape on Agilent TapeStation 2200 instrument and the Qubit, respectively. (Stopping point: Libraries can be stored at -80°C)
12. The libraries are now ready for sequencing. We recommend 30 million reads for each sample to get the appropriate sequencing depth. We used High Output 150 cycle kit (paired-end reads, single index) on the Illumina NextSeq 500 platform.
13. RNA-Seq reads should be processed by standard methods to identify RNAs that are enriched or depleted from the stress granule RNA in statistically meaningful manners.

2.2 Validate RNA-Seq results by single molecule FISH staining

To validate RNA-Seq results, we recommend using Stellaris single molecule FISH probes and buffers. Alternatively, probes can be made by making your own DNA oligos conjugated to fluorophores [21] and using Stellaris Buffers as described in this section. This section is an adaption of the Stellaris protocol (Sequential IF + FISH in Adherent Cells).

Instead of using U-2 OS cells expressing G3BP1-GFP, we recommend co-staining wild-type U-2 OS cells with G3BP1 antibody and the single molecule FISH probes. This approach leads to images with evenly distributed G3BP fluorescence between cells and facilitates image analysis by Imaris, an image analysis software.

2.2.1 Staining

1. Sterilize 18×18 1.5mm coverslips (Fisher Scientific, 12-541a) by incubating in 70% ethanol for 1' in 6-well tissue culture plates.
2. Aspirate the ethanol. Make sure coverslip and tissue culture plate are dry before seeding cells.
3. Seed U-2 OS cells at ~40% confluency in the 6-well tissue culture plates containing sterilized coverslips and grow overnight. Cells should be ~80% confluent by the next day.
4. Exchange media 60' before stress with fresh media. (Make sure media is at 37°C to ensure robust SG induction [19].)
5. Stress cells (See above).
6. Aspirate media. Wash cells with 1X PBS pre-warmed to 37°C . Aspirate PBS. Fix cells with 500 μL 4% paraformaldehyde for 10' at room temperature. (Stopping point: Cells can be stored after fixation in 1X PBS at 4°C)
7. Wash twice with 2 mL of 1X PBS.
8. Incubate cells in 2 mL of 0.1% Triton X-100 in 1X PBS for 5' at room temperature.
9. Wash once with 2 mL of 1X PBS.

10. Dilute mouse α -G3BP1 antibody in 1X PBS (5 μ g/mL final concentration, ab56574 (Abcam)). You will need 100 μ L of the diluted antibody per sample. Other antibodies that detect SGs robustly by immunofluorescence can be used instead.
11. Setup humidifying chamber. Flip coverslips with cells facing down onto the 100 μ L droplets (Figure 2).
12. Incubate for 60' at room temperature.
13. Transfer coverslips to a new 6-well plate containing 2 mL 1X PBS. Incubate for 10' at room temperature.
14. Repeat step 13 twice.
15. Dilute goat α -Mouse FITC-conjugated antibody in 1X PBS to 1:500 dilution (ab6785 (Abcam)). You will need 100ul of the diluted antibody per sample.
16. Flip coverslips with cells facing down onto the droplets as in step 11 (Figure 2).
17. Incubate for 60' at room temperature.
18. Transfer coverslips to a new 6-well plate containing 2 mL 1X PBS. Incubate for 10' at room temperature.
19. Repeat Step 18 twice.
20. Add 500 μ L 4% paraformaldehyde and incubate for 10' at room temperature.
21. Wash with 2 mL 1X PBS twice.
22. Aspirate 1X PBS off the coverslips.
23. Add 1 mL of Wash Buffer A and incubate at room temperature for 5'.
24. Prepare hybridization buffer containing single molecule FISH probes. Add 2 μ L of probe stock solution to 200 μ L of hybridization buffer. Mix well. This is sufficient for one coverslip.
25. Flip coverslips with cells facing down on 200 μ L droplets containing single molecule FISH probes (From Step 24) in a humidified chamber (Figure 2).
26. Seal humidified chamber with parafilm.
27. Incubate at 37°C for 16h in the dark.
28. Transfer coverslips to a new 6-well plate containing 2 mL of Wash Buffer A.
29. Incubate at 37°C for 30' in the dark.
30. Aspirate Wash Buffer A. Add fresh Wash Buffer A and incubate at 37°C for 30' in the dark again.
31. Aspirate Wash Buffer A. Add 2 mL of Wash Buffer B. Incubate for 5' at room temperature.

32. Add a small drop of Vectashield mounting medium with DAPI (H-1200, Vectorlabs) onto a microscope slide. Mount coverslip onto the slide with cells side down.
33. Seal coverslip with clear nail polish. (Stopping point: slides can be stored at 4°C in the dark up to 1 month)
34. Proceed to imaging.

2.2.2 Imaging parameters

We routinely use a wide-field microscope for imaging fixed cells stained with smFISH probes. We have also successfully performed the same protocol using standard laser scanning confocal microscopes. Below provides a starting point using a wide-field deconvolution microscope for imaging U-2 OS cells co-stained for G3BP1 and smFISH probes. Imaging parameters will need to be adjusted according to the type of microscope and staining protocol used.

1. Image fixed cells using a wide-field DeltaVision Elite microscope with a 100X objective using a PCO Edge sCMOS camera with appropriate filters.
2. 25 Z-sections for each field of view were imaged. The step sizes are 0.2 μm . (This allows us to capture the entire U-2 OS cell).
3. Imaging parameters were adjusted to restrict fluorescence within the scope's dynamic range. (We recommend imaging cells that were not stained with single molecule FISH probes to assess the degree of autofluorescence).
4. Deconvolve images with built-in DeltaVision software using the following parameters. Deconvolution Method: Enhanced Ratio (aggressive). Number of cycles: 10. Maximum Number of CPUs to use: 4. Camera Intensity Offset: 50. And with the following checked off: Apply correction, normalize intensity, and use photosensor.

2.2.3 Image analysis with Imaris

Imaris is an image analysis software from Bitplane. It has tools to segment cells and interprets 3D images. These tools can allow for precise quantification of single molecule FISH spots in cells and within SGs. Described below is a workflow on how to do this (Figure 3). The key steps are to mask the nuclei, manually segment individual cells, quantify the number of spots in a cell, and quantify the number of spots in SGs within a cell. Although we prefer Imaris, this can be done with other software including ImageJ with FIJI plugin (<http://imagej.net/Fiji>).

Load images in Imaris. (Imaris will render all the individual z-plane images in 3D. Imaris can read images and parameters of many different file formats including *.tif, *.tiff, *.dv, *.oib., and *.ism.)

2.2.3.1 Mask Nuclei

1. Click on ImarisCell creation wizard.

2. In ImarisCell creation wizard, select Cell with no nuclei.
3. Then select cytoplasm icon.
4. Select channel corresponding to the nuclei (DAPI staining) as the source channel.
5. Subsequently, select 0.5 μm filter width and manually determine threshold (Imaris will be rendering each step of the process so the user can assess if it is capturing the nuclei).
6. Select an adequate number of voxels above to account for all the nuclei in the image and end the ImarisCell creation wizard.
7. After completion of the ImarisCell wizard, we need to convert the cell rendered nuclei into surface. The surface creation wizard allows for masking. To do this, select the edit tab within the newly created ImarisCell module.
8. Select convert to surface.
9. A new surface creation module is created. Click on that, and select the edit tab.
10. Under mask properties, select mask all button. Now set voxels for all channels inside surface to 0. This will create new mask channels.
11. In the Display adjustment, unclick the original channels but not the masked channels. You should see cells with nuclei masked (Compare Figure 3A to 3B).

2.2.3.2 Segment individual cells

1. In the second step, we will manually segment individual cells. First segment cells by creating a ROI around a single cell using the surface creation wizard (For more information, see <http://www.bitplane.com/learning/masking-properties-of-imagis-tutorial>)
2. After creating the surface. Select the edit tab. Under mask properties, mask all channels outside surface to 0.
3. In the Display adjustment tab, hide original channels and the nuclei-masked channels. You should now see the one cell you manually segmented with the nuclei masked (Compare Figure 3C to Figure 3B).

2.2.3.3 Quantify the number of single molecule FISH spots

1. Once one has properly segmented a cell and masked the nuclei, the number of single molecule FISH spots in SGs and a cell can be counted.
2. To count the number of single molecule FISH spots in a cell, select spot creation wizard and use these parameters: 0.200 μm diameter and manually determined threshold on the properly masked single molecule FISH source channel (Compare Figure 3C and 3D).
3. After completion of spot creation wizard, the results are tabulated in excel format and can be retrieved in the spot created module.

2.2.3.4 Quantify the number of single molecule FISH spots in SGs

1. To count the number of fluorescent single molecule FISH spots in SGs, select ImarisCell creation wizard and apply these parameters on properly masked G3BP1 fluorescent source channel: 0.0406 μm filter width, manually-determined thresholding, 1 voxel, and properly masked single molecule FISH source channel: 0.200 μm diameter with manually determined thresholding.
2. After completion of ImarisCell Creation wizard, the results are tabulated in excel format and can be retrieved in the ImarisCell module.
3. Repeat Sections 3.3.2 to 3.3.4 to count single molecule FISH spots inside and outside SGs in other cells

3.1 Discussion of equipment

3.1.1 Cells

We used human osteosarcoma U-2 OS cells stably expressing G3BP1-GFP [22] and untagged U-2 OS cells (Nancy Kedersha and Paul Anderson). U-2 OS cells were maintained at 37°C/5% CO₂ in DMEM with high glucose, 10% fetal bovine serum, and 1% penicillin/streptomycin.

3.1.2 SG lysis buffer

We recommend making the solution fresh before lysing cells.

Recipe: 50 mM TrisHCl pH 7.4, 100 mM KOAc, 2 mM MgOAc, 0.5 mM DTT, 50 $\mu\text{g}/\text{mL}$ Heparin, 0.5% NP40, complete mini EDTA-free protease inhibitor (1 tablet/50 mL lysis buffer, 11836170001, Sigma-Aldrich), 1 U/ μL RNasein Plus RNase Inhibitor (N2615, Promega).

3.1.3 Equilibrated DEPC-treated Dynabeads

200 μL of Dynabeads (10002D, Thermo Fisher)/sample are needed for each SG isolation. We use 120 μL of Dynabeads for two pre-clearing steps and 60 μL for immunoprecipitation for each sample. All steps in this protocol are done at 4°C. Equilibrated DEPC-treated Dynabeads are made fresh for each experiment.

1. To equilibrate and DEPC-treat Dynabead, transfer required amounts of Dynabeads to a microcentrifuge tube.
2. Separate Dynabeads from storage solution using a magnet.
3. Aspirate and re-suspend Dynabeads in 1 mL of PBS containing 1 μL DEPC.
4. Nutate for 60' at 4°C.
5. Separate Dynabeads from solution again using a magnet.
6. Aspirate and wash with 1 mL of PBS.
7. Separate Dynabeads from wash buffer. Wash three more times but with SG lysis buffer.

8. Resuspend Dynabeads in 200 μ L SG lysis buffer and store at 4°C.

3.1.4 Wash Buffer 1

Recipe: 20 mM Tris HCl pH 8.0, 200 mM NaCl, and 1 U/ μ L of RNasein Plus RNase Inhibitor

3.1.5 Wash Buffer 2

Recipe: 20 mM Tris HCl pH 8.0, 500 mM NaCl, and 1 U/ μ L of RNasein Plus RNase inhibitor

3.1.6 Wash Buffer 3

Recipe: SG lysis buffer with 2M Urea

3.1.7 1X Proteinase K buffer

Recipe: 100 μ g/mL Proteinase K, 2M Urea, 1X TE buffer

3.1.8 Kits used for DNase treating samples and Ribo-depleting RNA

The kit we used to DNase treat the RNA is DNA-free DNA removal kit (AM1906, Thermo Fisher Scientific). The kit we used to deplete rRNA is Ribo-Zero rRNA removal kit (MRZH11124, Illumina).

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Highlights

1. SG cores can be biochemically purified by successive centrifugation and immunoprecipitation
2. Validation of RNA-seq results by single molecule FISH
3. Image analysis for single molecule FISH using the Bitplane Imaris image analysis software

Figure 1. Isolation of mammalian SG core RNA for RNA-Seq analysis

Schematic illustrating protocol for RNA isolation from SG cores. See Section 2.1 for a step-by-step guide.

Figure 2. Cartoon schematic on how to setup humidifying chambers for hybridizing with antibodies or single molecule FISH probes

The containers we used are square Petri dishes (FB0875711A, Fisherbrand). For overnight hybridization with single molecule FISH probes, we suggest sealing the container with parafilm to prevent probe solution from evaporating.

Figure 3. Flowchart of image analysis using Imaris Imaging Software for counting smFISH spots in single U-2 OS cells

(A) Sample image of U-2 OS cells stressed with NaAsO₂ stained for G3BP1 by immunofluorescence (Green fluorescence) and a specific RNA by single molecule FISH (Red fluorescence). Nuclei are stained with DAPI (Blue fluorescence). (B) Nuclei masked image. (C) Single cell image as shown in the boxed image from B. (D) Rendering of single molecule FISH spots by Imaris (White balls). (E) Rendering of SGs (Green), single molecule FISH spots outside SGs (White balls) and single molecule FISH spots inside SGs (Red balls). Scale bar 3 μ m.