A multicolor riboswitch-based platform for imaging of RNA in live mammalian cells

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Abstract

A key approach to investigating RNA species in live mammalian cells is the ability to label them with fluorescent tags and track their dynamics in the complex cellular environment. The growing appreciation for the diversity of RNAs in nature, especially the roles of small, non-coding RNAs for cell function, calls for development of orthogonal RNA tagging systems. We previously developed Riboglow, a new RNA tagging system that features modular elements and hence the possibility to customize features for each application of choice. Riboglow consists of an RNA tag that is genetically fused to the RNA of interest and a small molecule that binds the RNA tag and elicits a fluorescence light up signal. Here, we present an overview of the Riboglow platform and compare and contrast the system with existing RNA tagging systems. Two step by step protocols for implementation of RNA imaging with Riboglow in live mammalian cells are presented, with special emphasis on guidelines that drive choices for modular elements in the Riboglow platform. Modular elements in the Riboglow platform include the RNA tag sequence and size, the number of RNA tag repeats per tagged RNA, the fluorescent color of the probe, the identity of the chemical linker in the probe, and the concentration of the probe used in live cells. Together, Riboglow is a new RNA tagging platform that enables robust live cell imaging of RNA dynamics, and this detailed protocol and guidelines for implementation will enable broad usage of Riboglow.

Keywords

live cell microscopy; fluorescent RNA tags; stress granules; U-bodies; non-coding RNA tracking; single cell fluorescence microscopy; mRNA localization

Introduction

The growing appreciation of diverse roles for coding and non-coding RNAs in cell biology necessitates new tools and approaches to visualize RNAs and their dynamics in living cells (Pitchiaya, Heinicke, Custer, & Walter, 2014). A conceptually straightforward approach to illuminate an RNA of interest is to genetically fuse a fluorescent RNA tag to the RNA of interest (Urbanek, Galka-Marciniak, Olejniczak, & Krzyzosiak, 2014), akin to fluorescent protein fusions. In contrast to the protein world where fluorescent tags are derived from...
naturally occurring fluorescent proteins (FPs) (Rodriguez et al., 2017), no naturally fluorescent RNA species have been discovered. Hence, fluorescent labeling of RNA has necessitated different approaches to engineer a fluorescence signal onto an RNA tag.

Fluorescent RNA tags commonly consist of two elements, (i) the RNA tag sequence that is fused to the RNA of interest, and (ii) a protein or small molecule that binds to the RNA tag and induces a fluorescence signal. The most widely used fluorescent RNA tag is the bacteriophage-derived MS2 system, developed by the Singer lab (Fusco et al., 2003). In the MS2 system, the RNA tag consists of an array of MS2 stem-loop sequences (typically 12 to 24 repeats per tag) that are fused to the RNA of interest. Each stem-loop repeat sequence binds a dimer of the MS2 coat protein (MCP), which is genetically fused to a fluorescent protein (MCP-FP). The MS2 system allows for robust tagging of mRNAs on the single RNA level and studying the mRNA life cycle in mammalian cells (Tutucci, Livingston, Singer, & Wu, 2018). While highly useful for tracking mRNA species, the size of the MS2 tag (>1,000 nucleotides (nt) for the RNA tag, >80 kDa for MCP-GFP, assuming 24 copies) makes it incompatible for tagging small, non-coding RNAs, as these RNA species themselves may be much smaller in size. Therefore, orthogonal fluorescent RNA tags for labeling and tracking small non-coding RNA dynamics are needed to complement the MS2 system as the gold standard for mRNA visualization.

A number of small-molecule based fluorescent RNA tags were developed in response to the need for orthogonal systems. This approach was pioneered by the Jaffrey lab with development of Spinach (Paige, Wu, & Jaffrey, 2011) and subsequently Broccoli (Filonov, Moon, Svensen, & Jaffrey, 2014) and Pepper (J. Wu et al., 2019) aptamers. Similar approaches yielded the Mango (Autour et al., 2018; Dolgosheina et al., 2014), and the recently developed Peppers (Chen et al., 2019). In each case, a short RNA tag was evolved in vitro to bind to a small molecule that is non-fluorescent in the absence of the RNA, but induces fluorescence upon binding the RNA. Because these fluorescent RNA tags are not derived from naturally occurring RNA sequences, unwanted RNA processing (Filonov, Kam, Song, & Jaffrey, 2015) and binding of the small molecule to RNAs with similar structures (Jeng, Chan, Booy, McKenna, & Unrau, 2016) were noted complications. While further engineering of these platforms (Li, Kim, Litke, Wu, & Jaffrey, 2019; J. Wu et al., 2019) improve usability for RNA visualization, the diversity of RNA species in nature calls for broad exploration of orthogonal fluorescent RNA tagging systems.

Overview of the Riboglow RNA tagging platform

We previously developed the Riboglow platform as a tool that is complementary to existing RNA imaging approaches and enables tagging and tracking of RNAs and their dynamics in live cells (Braselmann et al., 2018). The platform consists of an RNA tag that is genetically fused to an RNA of interest and a small organic molecule that binds the RNA tag to elicit a fluorescence signal (Fig. 1). The RNA tag is derived from a bacterial riboswitch, namely the Cobalamin (Cbl) riboswitch (Johnson, Reyes, Polaski, & Batey, 2012), and the small molecule is a synthetic Cbl-fluorophore probe. Cbl itself has fluorescence quenching properties (Lee & Grissom, 2009), resulting in dim fluorescence of the Cbl-fluorophore probe in the RNA-unbound state. Upon binding to the riboswitch RNA, fluorescence
increases, modulated by the nature of the organic linker connecting Cbl and the fluorophore, and by the photophysical properties of the fluorophore (Braselmann et al., 2018). Importantly, the Cbl riboswitch consists of a large class of sequences in nature (Nahvi, Barrick, & Breaker, 2004), thus allowing usage of a series of RNA sequences that vary in length and ability to increase fluorescence upon binding the probe (Braselmann et al., 2018). We demonstrated that different Cbl-fluorophore probes with variable chemical linkers and variable fluorophores and different RNA tag sequences are compatible with visualizing RNAs in live cells (Braselmann et al., 2018). Together, we developed a versatile platform with modular features that can be selected based on each RNA imaging application to allow for robust tagging and tracking of RNAs in live mammalian cells.

A key feature of the Riboglow platform is the modularity of individual elements. Several features of the platform are variable and therefore allow for customization, including choice of the RNA tag sequence (and hence length of the RNA tag), number of RNA tag repeats per RNA, choice of Cbl-fluorophore probe with different linker and fluorophore properties and optimization of probe concentration. While this versatility offers the opportunity to customize and hence optimize each RNA imaging experiment, care must be taken to consider how customization choices are made. Key steps that require customization are outlined below, with examples on how choices were rationalized in previous studies (Braselmann et al., 2018). Riboglow includes a genetically encoded RNA tag portion, allowing in principle for visualization of any RNA of interest that can be appended with the tag. We have implemented Riboglow for two distinct protocols that are outlined below, including visualization of mRNA dynamics and recruitment to stress granules (Braselmann et al., 2018) (protocol 1) and tagging of short non-coding U snRNA for which no other live cell RNA tag exists (Braselmann et al., 2018) (protocol 2).

Comparison of Riboglow with related techniques

Advantages

The Riboglow platform exhibits key advantages in comparison with the established MS2 system and the Broccoli system (Table 1). First, a key advantage of Riboglow is that the size of the tag is relatively small, even when 4 copies of Riboglow are used as a tag for one mRNA (Braselmann et al., 2018) (tag length = 430 nt, mass of 4 fluorophores = 2.3 kDa for 4x Riboglow, versus tag length = 102 nt, mass of 4 fluorophores = 176 kDa for 2x MS2-GFP). The need to minimize tag size becomes especially relevant when non-coding RNAs are tagged, where structural features may encode for functional elements (Fabbri, Gmira, Varani, & Calin, 2019). We showed that U1 snRNA can be visualized with just one copy of Riboglow (tag length = 81 nt, mass of 1 fluorophore = 2.3 kDa) (Braselmann et al., 2018).

Second, the Cbl-fluorophore probe binds the RNA tag via the Cbl moiety. Thus, probes with different colors can be used interchangeably and synthetic fluorophores with ideal photophysical properties (low photobleaching, excitation wavelengths that match laser lines and microscope setup) can be chosen. We validated Riboglow-ATTO590 and Riboglow-Cy5, where the fluorophores of choice were ATTO590 (λ<sub>ex</sub> = 594 nm, λ<sub>em</sub> = 622 nm) and Cy5 (λ<sub>ex</sub> = 646 nm, λ<sub>em</sub> = 662 nm), respectively (Braselmann et al., 2018). Third, we determined that Riboglow performs on par with the MS2 platform in a fluorophore-by-
fluorophore comparison when tagging mRNAs and visualizing their recruitment to stress granules. In contrast, tagging mRNA with Broccoli was insufficient to visualize stress granules (Braselmann et al., 2018) (Table 1). Together, our systematic and quantitative comparison of Riboglow versus the MS2 system and Broccoli serve as an ideal starting point to evaluate Riboglow’s potential to label and track diverse types of RNA.

**Limitations**

Despite the advantages of Riboglow in comparison with existing RNA imaging techniques, it is important to be aware of limitations when designing experiments for RNA imaging with Riboglow (Table 1). First, the Cbl-fluorophore probe is not cell-permeable. We explored various means of introducing the probe into live cells (electroporation, bead loading) and determined that bead loading is a technique that is robust and compatible with adherent cells in our assays (see ‘Precursor technique: Bead loading’ at the end of this chapter) (Hayashi-Takanaka et al., 2011; McNeil & Warder, 1987; Morisaki et al., 2016). While we find this technique straightforward to implement, it may not be compatible with every cell type of interest, as cells must be adherent, and exploring alternative options (including electroporation and microinjection of the probes) may be required. Second, sufficient contrast for live imaging is critical and can be achieved in different ways. One option is to repeat the number of RNA tags per RNA of interest. We systematically assessed contrast when visualizing recruitment of ACTB mRNA to stress granules (Braselmann et al., 2018) and showed that four copies of the RNA tag generate sufficient contrast for this application, whereas one copy was insufficient (Braselmann et al., 2018). It is important to note that while ACTB mRNA is a common model mRNA to visualize mRNA recruitment to stress granules (see for example (Zurla, Lifland, & Santangelo, 2011)), only <10% of all ACTB mRNA molecules accumulate in stress granules (Khong et al., 2017). Hence, contrast to visualize fluorescence signal of ACTB mRNA in stress granules over cytosolic background will include labeled mRNA that remain cytosolic. In cases where the RNA of interest localizes to RNA protein granules such as stress granules or U-bodies, as little as one copy of the RNA tag may be sufficient (as in the case for U-body detection (Braselmann et al., 2018)), because concentrating the RNA in granules increases contrast. For these applications the contrast will depend on the number of copies of the RNA of interest and the fraction that gets recruited to RNA-protein granules. Multiplexing the RNA tag increases the tag size, a feature one must consider when designing a Riboglow imaging experiment. Lastly, we have used Riboglow in applications where the RNA of interest was produced from a transiently transfected plasmid driven by a relatively strong promoter (such as a CMV promoter) (Braselmann et al., 2018). While we expect that production of the RNA at endogenous levels should be compatible with the Riboglow technique as well, we have not explicitly tested this. Together, it is important to consider these limitations when considering Riboglow as a platform to visualize an RNA of interest in live mammalian cells.

**Considerations for tagging RNA with Riboglow**

**Choice of RNA tag and number of repeats per RNA of interest**

Because the RNA tag portion of Riboglow is derived from the Cbl riboswitch, many different RNA variants are available in phylogeny (Nahvi et al., 2004) to use as the RNA tag.
We tested five Cbl riboswitch variants for binding to the Cbl-fluorophore probe and found that all sequences tested induce fluorescence signal increase in vitro (Braselmann et al., 2018). In principle, all five sequences tested may be used for tagging an RNA of interest, although we choose one for cellular assays (variant A and AT, a truncated version of A, (Braselmann et al., 2018)). Parameters to consider in choosing a tag sequence are (i) overall length of the tag sequence (ranging from 81 to 130 nts for a single tag (Braselmann et al., 2018)) and (ii) affinity for the Cbl-fluorophore probe (K_D values ranging from μM to nM, (Braselmann et al., 2018)). Depending on the RNA of interest and the application, attaching multiple copies of the RNA tag may be advantageous to increase cellular contrast. We have previously quantitatively compared 1, 2 and 4 copies when visualizing ACTB mRNA recruitment to stress granules (Braselmann et al., 2018). Thus, while a large set of Cbl riboswitch sequences are available in principle to use as the RNA tag portion, we recommend using variant A or AT, which we previously validated in cellular assays.

Choice of Cbl-fluorophore probe

Because the RNA tag binds the Cbl portion of the Cbl-fluorophore probe (Fig. 1), the fluorophore and chemical linker connecting the two may be varied without interfering with the RNA/Cbl interactions that determine binding. We systematically assessed performance of a series of 9 different Cbl-fluorophore probes with a total of 6 different linkers and 5 different fluorophores (Braselmann et al., 2018). Three of the probes were used in various applications in live cell RNA visualization assays (Cbl-Cy5, Cbl-4xGly-ATTO 590, Cbl-5xPEG-ATTO 590, Table 2) (Braselmann et al., 2018). When choosing a probe for a new application, one should consider fold fluorescence increase of the probe alone vs. RNA-bound probe (determined in vitro in (Braselmann et al., 2018), Table 2), assessment of non-specific localization of the probe in the cell line of interest (Table 2) and compatibility with existing fluorescent labels. For example, we determined that all three probes localize largely diffusely throughout the cytosol of U-2 OS cells. In contrast, while Cbl-4xGly-ATTOS90 and Cbl-5xPEG-ATTO590 localize diffusely throughout the cytosol of HeLa cells, Cbl-Cy5 localizes to cytosolic puncta in this cell type (Braselmann et al., 2018) (Table 2). Together, several Cbl-fluorophore probes are available for RNA imaging, and we recommend consultation of features listed in Table 2 when choosing a probe.

Choice of probe concentration

We find that optimization of probe concentration for each application improves robust detection of a desired RNA of interest, and that photophysical properties affect usable concentrations of each probe. For example, we found that a Cy5-based probe can be used at lower concentrations due to brightness of Cy5 and high signal over cellular background. These features make Cy-based probes compatible with integrating the fluorescence signal over several seconds during image acquisition, which may be a useful feature to improve signal over background, especially when RNAs are not mobile in this time frame. Using low probe concentrations and improving contrast by signal integration may be advantageous when non-specific localization of the probe is a concern and needs to be reduced as much as possible, as we have demonstrated for mRNA localization to stress granules (Braselmann et al., 2018). In contrast, low RNA concentrations for tracking of dynamics may require higher probe concentrations to achieve sufficient binding of the probe to the RNA. In addition,
image processing and quantification may require the fluorescence signal to be within a defined range. We recommend establishing an image processing pipeline using pilot experiments to determine whether image processing dictates a certain signal intensity and hence probe concentration (see below for a detailed discussion). Together, we recommend empirical optimization of probe concentrations for each assay, guided by experiments we performed previously (Table 2).

Considerations for timing of experiment

Timing of experimental steps in executing the imaging protocol are critical and depend on the application of choice. Generally, experiments consist of (i) introducing a plasmid to produce the Riboglow-tagged RNA to cells, (ii) loading the Cbl-fluorophore probe to cells, and (iii) performing live cell imaging. When the Riboglow-tagged RNA is produced transiently from a plasmid, high levels of the RNA will accumulate about 24 hours post transfection, a desirable feature for stress granule imaging and U-body visualization (Braselmann et al., 2018). Additionally, we determined that the Cbl-fluorophore probe accumulates non-specifically in aggregates in cells, in particular in HeLa cells (especially for Cbl-Cy5, see Table 2) and when no RNA binding partner is present. This non-specific accumulation is most pronounced about 6–7 hours after introduction of the probe (Braselmann et al., 2018). Therefore, we recommend loading the Cbl-fluorophore probe no earlier than 1–2 hours prior to imaging to prevent non-specific aggregation, if the desired imaging application permits this timeline. We recommend appropriate control experiments to avoid imaging artifact localization patterns due to non-specific probe localization when experiments on a longer timeline are necessary.

Considerations for data processing and analysis

It is critical to consider image processing and analysis pipelines when optimizing experimental conditions. We have demonstrated RNA detection using the Riboglow platform for two different RNA imaging experiments that require distinct image processing procedures (Braselmann et al., 2018). First, we visualized recruitment of mRNAs to stress granules (step by step protocol 1, below). In this application, we quantified colocalization of Cbl-fluorophore signal with a stress granule marker protein (Braselmann et al., 2018). Because we used the ratio of the fluorescence signal in each granule over cytosolic background to assess accumulation of the Riboglow signal at the granule, cells with very high or very low probe concentrations must be excluded to avoid artifacts. Because the probe concentration varies between different cells due to bead loading heterogeneity, one must ensure that cells with the same concentration range are used in experimental and control conditions (Fig. 2) (Braselmann et al., 2018). Second, we have visualized U1 snRNA recruitment to cytosolic U-bodies (step by step protocol 2, below). In this case, we analyzed cells by determining whether cytosolic granules (U-bodies) were present or not for experimental or control conditions, and compared incidence of these granules across conditions (Braselmann et al., 2018). We found that it is key to collect images for many cells in a short period of time to limit other biological effects that may affect cell physiology and hence non-specific probe localization, and to ensure that timing of each step is the same for experimental and control conditions. Similar to the stress granule experiment, it is critical to
ensure that final probe concentrations in cells are comparable between experiment and controls.

**Materials, equipment and reagents**

**Cell lines and cell culture**

Adherent U-2 OS cells (ATCC® HTB-96™) or HeLa cells (ATCC® CCL-2™) were used for all experiments described in this chapter. To allow for labeling the stress granule marker protein G3BP1, a variant of U-2 OS cells, U-2 OS Halo-G3BP1, was used where a Halo-tag was added to G3BP1 in the genomic locus by CRISPR editing. Generation of this cell line is described in (Braselmann et al., 2018). Alternatively, a U-2 OS cell line where G3BP1 tagged with a fluorescent protein is stably integrated in the genome may be used (such as GFP-G3BP1, described in (Braselmann et al., 2018)).

Cells should be maintained and subcultured using standard cell culture protocols and procedures, as recommended by ATCC®. We maintain cells in Dulbecco’s modified eagle medium (DMEM, Gibco) supplemented with 10% FBS (Gibco) at 37°C/5% CO₂. To avoid contamination, we do not add antibiotics routinely. We recommend using cells only up to passage 12–15 upon receipt from ATCC® or verification of the genetic modification to avoid cross-contamination of cell lines.

**Plasmids**

Plasmids to produce Riboglow-tagged ACTB mRNA were constructed by standard molecular cloning techniques and are described in detail in (Braselmann et al., 2018). All plasmids described in this chapter including plasmid maps are available from Addgene or from the authors (Table 3).

**Cbl-fluorophore probes: General information, storage and handling**

All Cbl-fluorophore probes in (Braselmann et al., 2018) were synthesized in the laboratory of Dorota Gryko, Polish Academy of Sciences, Poland. Samples of the following probes in powder form are available upon request from the authors of this chapter: Cbl-5xPEG-ATTO 590, Cbl-4xGly-ATTO 590, Cbl-Cy5. These samples are sufficient for a few pilot experiments (~20 imaging dishes). Protocols with details on how to synthesize these probes and other probe variants are provided in reference (Braselmann et al., 2018).

The probes (in powder form or as stock solutions) should be stored in the dark at −20°C. While it is preferable to store the dry powder, we have not found adverse effects for imaging results when working with probe stock solutions in cell culture grade DMSO that were stored in the dark at −20°C for several weeks. Each probe in powder form should be brought up in a small volume of DMSO (15–20 μL) in the original tube to avoid loss of material. Vigorous mixing by vortexing ensures that all material goes into solution. The minimal volume of added DMSO ensures that the master stock is sufficiently concentrated. To determine the concentration of the stock solution in DMSO, one should prepare 500 μL of a 1:500 – 1:1,000 dilution of the stock in RNA buffer (100 mM KCl, 10 mM NaCl, 1 mM MgCl₂, and 50 mM HEPES, pH 8.0) or phosphate buffered saline (PBS). The absorbance
should be determined at the indicated wavelength (Table 3) in a 1 cm path length quartz cuvette using a UV-VIS spectrophotometer. After subtracting the buffer blank, the probe concentration in the master stock can be calculated using the extinction coefficient listed in Table 3, and considering the dilution factor. The master stock should be stored at −20°C, and a fresh stock in PBS from the master stock should be prepared on the day of each experiment.

**Halo-tag labeling dyes**

To label the Halo-G3BP1 marker protein, dyes from the Janelia Fluor® (JF) family functionalized for Halo tag labeling are used. These dyes can be obtained from Luke Lavis (Janelia Research Campus) (Grimm et al., 2017). All dyes used in this chapter are HaloTag ligands, referred to as JF\(_{585}\), SiR\(_{594}\) (both compatible with common red fluorescence microscopy filter settings and orthogonal with Cbl-Cy5) and JF\(_{646}\) (compatible with common far red microscopy filter settings, and orthogonal with ATTO 590 Cbl-fluorophore probes) (Braselmann et al., 2018). Handling of probes and storage was done according to recommendations by Luke Lavis.

**Buffers, reagents and kits**

- U-2 OS cells (ATCC\(^{®}\) HTB-96\(^{™}\))
- HeLa cells (ATCC\(^{®}\) CCL-2\(^{™}\))
- U-2 OS Halo-G3BP1 cells (Braselmann et al., 2018)
- Dulbecco’s modified eagle medium (DMEM, Gibco) for culturing cells
- Fetal bovine serum (FBS) (Gibco) for culturing cells
- 0.25% Trypsin EDTA (Gibco) for culturing cells
- RNA buffer (100 mM KCl, 10 mM NaCl, 1 mM MgCl\(_2\), and 50 mM HEPES, pH 8.0), autoclave salt components and sterile filter final buffer
- phosphate buffered saline (PBS)
- sodium (meta) arsenite (90%, S7400, Sigma-Aldrich), refer to safety information of the manufacturer and follow guidelines for safe handling. Handle the powder in the chemical hood and dispose of liquid waste according to safety regulations. Make 50 mM sodium arsenite stocks in PBS fresh on the day of each experiment.
- Cbl-fluorophore probes (Cbl-5xPEG-ATTO 590, Cbl-4xGly-ATTO 590, Cbl-Cy5), available in powder form from the authors of this chapter
- Janelia Fluor® (JF) dye variants for Halo tag labeling (Grimm et al., 2017) (abbreviated in this chapter as JF\(_{585}\), SiR\(_{594}\), JF\(_{646}\)), available from Luke Lavis (Janelia Research Campus)
- TransIT®-LT1 Transfection Reagent (Mirus)
- Opti-MEM® Reduced-Serum Medium (Thermo Fisher), use with TransIT®-LT1 Transfection Reagent kit according to protocol
• Plasmid Midi Kit (Qiagen), and reagents required within the kit (100% Ethanol, LB media for bacterial culture)
• imaging media (FluoroBrite DMEM, Thermo Fisher)
• 100% ethanol to clean beads
• 2 M NaOH to clean beads
• Desiccant to store beads (for example W.A. Hammond Drierite™ Indicating Absorbents, Fisher Scientific)
• Thapsigargin (VWR), 10 μM aliquots in DMSO

**Equipment**

• Cell culture equipment: common biosafety cabinet and incubator, accessories for culturing U-2 OS and HeLa cells (U-2 OS cells are BSL-1 and HeLa cells are BSL-2).
• Confocal laser scanning microscope with 561 nm and 638 nm laser lines, including appropriate filters, 100x oil objective (1.45 NA, Plan Apo I) and environmental chamber to maintain cells at 37°C/5%CO₂ during image acquisition. We use a Nikon A1R Laser Scanning Confocal Microscope.
• Cell culture dishes for live cell imaging (imaging dishes), 35 mm diameter. We use home-made imaging dishes with a ~10 mm center hole covered by cover glass (No. 1.5, VWR). Similar dishes are commercially available (for example MatTek; P35G-1.5–10-C).
• Hemocytometer for cell counting.
• Glass beads (106 μm; Sigma-Aldrich; G-4649) for bead loading. See Precursor technique at the end of this chapter for assembly of beads and nylon mesh filter as a bead loading device. Wear gloves and work under a hood when handling glass beads.
• Nylon mesh filter for bead loading device (Nylon Screen, 3×3”, 100 Mesh, Product No. 41–3110, Ted Pella, Inc.)

**Step by step protocol 1: mRNA localization to stress granules in U-2 OS cells**

A step by step protocol to visualize recruitment of β-actin mRNA to stress granules is provided below (Fig. 3). As we determined previously (Braselmann et al., 2018), four copies of the Riboglow tag (variant A) should be fused to the mRNA for sufficient contrast when visualization β-actin mRNA (see Table 3 for plasmid information).

**Day 1: Seed cells**

Seed U-2 OS Halo-G3BP1 cells in imaging dishes (35 mm diameter). Each imaging dish should have a seeding density of 0.25×10⁶ cells. Prepare four imaging dishes (Table 4).
Alternatively, U-2 OS GFP-G3BP1 cells can be used, where G3BP1 is labeled with GFP (Braselmann et al., 2018).

**Day 2: Transfection**

Plasmids used previously (Braselmann et al., 2018) and in this chapter are listed in Table 3 along with information to acquire them. Use plasmid DNA stocks that were prepared to high purity and high concentration (>1 μg/μL), for example with the Qiagen Plasmid Midi Kit. DNA stocks should be diluted to a final concentration of 1 μg/μL. Use chemical transfection with the TransIT®-LT1 Transfection Reagent (Mirus) and follow the manufacturer’s recommendation. Perform transfections of four imaging dishes (experimental dish plus appropriate controls) according to Table 4. For each transfection reaction, mix 1 μL of each plasmid DNA (add water yielding 2 μL volume per reaction, if needed), plus 250 μL Opti-MEM®, plus 6 μL TransIT®-LT1 Reagent and mix thoroughly. Incubate the mixture at room temperature for 15 min before adding to the imaging dish. While transfection efficiency with this method is low for U-2 OS cells (typically 20–25%), we found that this protocol results in sufficiently high expression levels of the mRNA for robust imaging. Perform the transfection such that imaging will occur 24 h post transfection.

Co-transfecting the plasmid NLS-TagBFP serves as a transfection marker, as we found that co-transfection efficiency of the plasmid encoding for the Riboglow-tagged ACTB mRNA and the plasmid encoding for NLS-TagBFP is >90% (Braselmann et al., 2018). Thus, it is possible to identify cells that are positive for the Riboglow-tagged ACTB mRNA with >90% accuracy. As an alternative approach, it may be advantageous to include a gene encoding for a fluorescent marker protein on the same plasmid encoding for the tagged mRNA.

**Day 3: Imaging**

Cells are first incubated with the appropriate Janelia Fluor® (JF) dye to label G3BP1, the stress granule marker protein. A Halo-tag is fused to the marker protein G3BP1, which reacts with JF dye variants (Grimm et al., 2017) (abbreviated as JF585, SiR594, JF646 throughout this chapter). Use a dye with fluorescent properties orthogonal to the Cbl-fluorophore probe (Table 2 for JF dye / Cbl-fluorophore combinations). Store JF dye stock solutions at −20°C at 100 μM in DMSO. For each dish, prepare 1 mL cell culture media (DMEM/5% FBS) and supplement with a final concentration of 1 μM JF dye (Braselmann et al., 2018). Note that we found recently that the JF dye concentration can be reduced ~100fold for this assay. Replace the media in the imaging dish with media containing JF dye and incubate at 37°C/5% CO₂ for 20 min for staining. Wash out unbound dye by replacing the media first with PBS and then with DMEM/5% FBS.

Bead load the desired Cbl-fluorophore probe, as described in the precursor technique at the end of this chapter (see also (Hayashi-Takanaka et al., 2011; McNeil & Warder, 1987; Morisaki et al., 2016)). Prepare a small aliquot of the desired Cbl-fluorophore probe in PBS for loading (3 μL of this solution are needed per imaging dish) (recommended concentrations for each probe are listed in Table 2). The volume of the Cbl-fluorophore probe to bead load may be increased to improve efficiency (we have had success increasing the volume up to 20 μL).
Stress granule formation is initiated by treating cells with 0.5 mM sodium arsenite. To reduce timing between probe loading and imaging, use imaging media supplemented with 0.5 mM sodium arsenite as the media added to cells at the last step in the bead loading protocol after rinsing beads off cells. Incubate cells at 37°C/5% CO$_2$ for stress granule formation for 30 min. We collected images for the stress granule assay 30 – 60 min after arsenite treatment, as prolonged arsenite treatment leads to cell death.

Live cell fluorescence microscopy can be done on any fluorescence microscope with the appropriate filter settings, although we recommend a laser scanning microscope over a widefield microscope because fluorescence contrast between stress granule and cytosolic signal is sharper for laser scanning microscopy. We used a Nikon A1R Laser Scanning Confocal Microscope with a 100x oil objective (1.45 NA, Plan Apo I). Laser lines used were 405 nm (for NLS-TagBFP imaging), 488 nm (for GFP-G3BP1), 561 nm (for ATTO 590 in Cbl-fluorophore probes and for JF dyes JF$_{595}$ and SiR$_{594}$) and 638 nm (for Cbl-Cy5 and JF dye JF$_{646}$). All live images in reference (Braselmann et al., 2018) were acquired with an environment chamber at 37°C.

**Image processing**

To quantify localization of β-actin mRNA tagged with Riboglow to stress granules, we have developed the following workflow (Fig. 2). Cells that were transfected with the Riboglow-tagged ACTB mRNA (ACTB-(A)4x) were identified via the NLS-TagBFP transfection marker, and cells that were bead loaded were identified via residual fluorescence retained from the Cbl-fluorophore probe. Only cells that had the NLS-TagBFP marker and were bead loaded were used for the analysis, except in the case of the untransfected control dish, where all cells positive for the probe were used for analysis. In an image processing software such as ImageJ, stress granules were identified in the Halo-G3BP1 channel. A line trace was drawn through each stress granule that included baseline cytosolic fluorescence near the granule (to account for cell to cell variability in probe uptake). The same line trace was recorded in the Cbl-fluorophore probe fluorescence channel. After subtracting extracellular background signal, the Cbl-fluorophore probe fluorescence trace as well as the control Halo-G3BP1 trace were plotted (Fig. 2). The maximum fluorescence signal for the Cbl-fluorophore probe was determined and divided by the average probe fluorescence in the cytosol (as noted in Fig. 2), yielding a fluorescence ratio (SG/cytosol). It is critical to do this analysis for the experimental set as well as an untransfected control, and it may be necessary to introduce a cutoff for fluorescence counts in this analysis and exclude cells with very low or very high probe concentrations. For example, the ratio will yield unreasonably high numbers when the fluorescence counts are close to the background (Fig. 2b). We always verified that the cytosol fluorescence counts in the Cbl-fluorophore channel are at a comparable level for the experimental and untransfected control sets.

The ratio analysis should be done for the experimental condition and appropriate controls (Table 4). Dish #2 (untransfected, loaded) will determine whether the probe accumulates in stress granules in a non-specific manner. Dish #3 (transfected, not loaded) will determine whether cellular background fluorescence contributes to apparent probe accumulation signal.
Dish #4 (transfected with marker only, loaded) will determine whether the transfection process alone recruits the probe to the granules.

**Step by step protocol 2: U1 snRNA localization to U-bodies in HeLa cells**

A step by step protocol to visualize formation of U-bodies via Riboglow-tagged U1 snRNA is provided below (Fig. 4). We determined previously (Braselmann et al., 2018) that U1 snRNA tagged with one copy of Riboglow variant A₇, a truncated minimal version of variant A, is sufficient to visualize U-bodies (see Table 3 for plasmid information).

**Day 1: Seed cells**

Seed HeLa cells in imaging dishes (35 mm diameter). Each imaging dish should have a seeding density of 0.1×10⁶ – 0.15×10⁶ cells. Prepare two imaging dishes (experimental and untransfected control).

**Day 2: Transfection**

Information to acquire a plasmid encoding for U1 snRNA tagged with Riboglow variant A₇ can be found in Table 3. Use a plasmid DNA stock that was prepared to high purity and high concentration (>1 μg/μL), for example with the Qiagen Plasmid Midi Kit. DNA stocks should be diluted to a final concentration of 1 μg/μL. Use chemical transfection with the TransIT®-LT1 Transfection Reagent (Mirus) and follow the manufacturer’s recommendation. Perform transfection of the experimental dish and include a control that is treated identically, but add water instead of plasmid DNA in the transfection procedure. An alternative control condition could be to transfect a plasmid encoding for a gene that does not bind the Cbl-fluorophore probe. For each transfection reaction, mix 1 μL of plasmid DNA (water for the untransfected control), plus 250 μL Opti-MEM®, plus 3 μL TransIT®-LT1 Reagent and mix thoroughly. Incubate the mixture at room temperature for 15 min before adding to the imaging dish. Perform the transfection such that imaging will occur 24 h post transfection.

**Day 3: Imaging**

Replace culture media in each dish with media supplemented with 10 μM Thapsigargin (10 mM aliquots in DMSO can be stored at −20°C) and incubate at 37°C/5% CO₂ for 3 h. After the 3 h incubation step, bead load the desired Cbl-fluorophore probe, as described in the precursor protocol at the end of this chapter (see also (Hayashi-Takanaka et al., 2011; McNeil & Warder, 1987; Morisaki et al., 2016)). At the last rinsing step of bead loading, add 1 mL cell culture media, supplemented with 10 μM Thapsigargin, and incubate at 37°C/5% CO₂ for an additional 30 min. Perform imaging experiment within 1 h of probe loading (see Fig. 4 for a recommended timeline). To minimize the potential for artifacts, we recommend not extending the incubation time at any step. It is advantageous to offset the procedure for the experimental and control dish by 30–60 min to make sure timing of each step is the same between conditions.

Live cell fluorescence microscopy can be done on any fluorescence microscope with the appropriate filter settings, although we recommend a laser scanning microscope over a
widefield microscope. As for stress granules, U-bodies appear as distinct puncta more easily by laser scanning microscopy when out of focus fluorescence signal is minimized. We used a Nikon A1R Laser Scanning Confocal Microscope with a 100× oil objective (1.45 NA, Plan Apo I). We used a 561 nm laser line (for Cbl-5xPEG-ATTO 590). All live images in reference (Braselmann et al., 2018) were acquired with an environment chamber at 37°C.

We collected images for U-bodies to up to 1 h after bead loading of the probe.

**Image processing**

We quantified formation of U-bodies by counting the number of cells with visible cytosolic granules. Consistent with other previous results in the literature (Tsalikis et al., 2015), we confirmed U-bodies in ~24% of HeLa cells, whereas just ~6% of untransfected control cells have cytosolic puncta (Braselmann et al., 2018) (Fig. 5). To avoid a bias in image acquisition (‘cherry picking cells’), we focused on one cell first and then collected images without inspecting the field of view (using the large image feature in the Nikon Elements software). Using this strategy, we can acquire images of a large area while avoiding bias in selecting areas with cells display phenotypes we expect. In total, we recommend performing at least three independent experiments, with the goal to have >300 cells total. To remove bias, it may be advantageous to perform a blind analysis, where files collected from the experimental and control condition are labeled in a non-descriptive way such that the person doing the analysis does not know which file belongs to which condition.

**Precursor technique: Bead loading**

Small molecules such as the Cbl-fluorophore probes can be introduced into live adherent cells by bead loading (Fig. 6) (Hayashi-Takanaka et al., 2011; McNeil & Warder, 1987; Morisaki et al., 2016). Briefly, media is aspirated from cultured cells, followed by adding a small volume of the probe and a layer of small glass beads directly onto the cells. Cell membranes are then briefly ruptured mechanically via beads to allow for entry of the probe into cells, followed by replacing the media. It is critical to perform this procedure rapidly (without rushing) to avoid exposure of the cells without media for too long.

**Preparations**

- Prepare glass beads for assembly in the bead loader as follows. Wash and sterilize beads by soaking in 2 M NaOH for 2 h and gently mixing using a shaker or rotator. Rinse beads extensively with distilled water until the pH becomes neutral, followed with rising in 100% ethanol for a few times. Air-dry beads overnight (or longer), by incubating at room temperature in the presence of desiccating material. Perform all steps in a hood to avoid inhaling beads. Washing steps should be performed by carefully aspirating each solution while avoiding removing the beads.

- Assemble glass beads in a ‘bead loader’ (Fig. 6) as follows. Punch a hole into a 35 mm culture dish in the center of the dish, sterilize by exposure to UV light, cover the hole with the nylon mesh (Nylon Screen, 3×3”, 100 Mesh, Product No. 41–3110, Ted Pella, Inc.) (use scotch tape to adhere the mesh to the bottom of the culture dish). Pour glass beads on the mesh, cover the dish with a lid, tape the
lid to the dish. The resulting bead loader should be stored in a larger container that includes desiccating material to ensure that the beads remain dry. Store the loader with a lid covering the mesh bottom and enclose in a container. The bead loader should remain in its container unless in use in a cell culture hood to avoid contamination, and to avoid exposure of beads to the air.

- Adherent cells should be cultured in imaging dishes (35 mm) to a confluency of 50–80%. Avoid too spare cell density as some cells detach during the procedure, and too dense confluency, as over-confluent cells may peel off in larger patches during loading.

- Prepare Cbl-fluorophore probe in PBS at the recommended concentration (Table 2).

- Ensure that solutions, media, pipettes and tips are prepared and ready in the hood, as the procedure should be performed with minimal breaks to avoid drying of the cells.

**Step 1**
- Aspirate all media off cells. Immediately add 3 μL of the Cbl-fluorophore solution to the cells.

**Step 2**
- Sprinkle a layer of glass beads on the center of the imaging dish using the bead loading device. The bead density should be high enough to cover the cells, while maintaining close to a single layer of beads.

**Step 3**
- Tap the imaging dish ~8 times forcefully on the cell culture surface to allow cell membrane rupture and loading. The force of tapping and number of taps may be adjusted for different cell types, depending on how adherent cells are. Bead loading is efficient when cells are forcefully tapped at this stage, but cells may also detach from the dish if the dish is tapped on the surface too hard. To optimize bead loading, we recommend loading a non-cell permeable fluorescent dye to avoid using the Cbl-fluorophore probe for optimization of this step. By using a fluorescent dye, one can easily assess bead loading by fluorescence microscopy. We typically achieve 40–50% loading efficiency for U-2 OS cells.

**Step 4**
- Immediate add 1 mL cell culture media and incubate at 37°C/5% CO₂ to recover cells for at least 5 min. Prior to imaging, rinse cells once in PBS to remove beads and excess probe and add appropriate culture media.
## Troubleshooting & Optimization

| Problem                                      | Solution                                                                                                                                 |
|----------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| No / few cells have Cbl-fluorophore probe.  | Optimize bead loading by varying cell density, probe concentration, mechanical loading step. Use a fluorescent dye that is not cell permeable to optimize the procedure. |
| Fluorescence signal from Cbl-fluorophore probe is dim. | Increase Cbl-fluorophore probe concentration (Table 3) and/or volume of stock added to cells (up to 20 μL per imaging dish).          |
| No / few cells have NLS-TagBFP transfection marker. | Optimize transfection efficiency of U-2 OS cells by varying cell density.                                                              |
| Riboglow recruitment to stress granules results in very high fluorescence ratio cytosol/granule (>15). | Signal is likely too dim and close to the background, select cells with higher probe concentrations.                                   |
| No mRNA at stress granules / no U-bodies detected. | Verify production of Riboglow-tagged RNA and colocalization with stress granules / U-bodies by fluorescence in situ hybridization (FISH). See (Braselmann et al., 2018) for protocols and probe sequences. Verify transfection of tagged RNA by producing reporter gene (fluorescent protein) from the same plasmid. |
| No cells with cytosolic granules detected in U-body assay. | Verify transfection by repeating with a transfection marker plasmid encoding for a fluorescent protein, determine transfection efficiency (should be >80% for HeLa cells). |
| Cytosolic granules detected in both experimental and control condition for U-body assay. | Reduce Cbl-fluorophore probe concentration to avoid non-specific probe aggregation. A control without Thapsigargin-treatment should not result in granule or aggregate formation. |

## Summary

We present a detailed protocol to implement RNA labeling and tracking in live mammalian cells with the Riboglow platform. A key feature that distinguishes Riboglow from similar techniques is the modular nature of the platform that offers flexibility for broad applicability of many different imaging systems. In particular, the Cbl-fluorophore probe portion of the platform is variable in that different fluorophores and hence colors and chemical linkers are available. Factors that influence what choices are best suited for different applications are discussed. Because the Cbl-fluorophore probe is not cell permeable, we have established bead loading as a technique to introduce the probe into live cells. While we find this technique relatively straightforward to implement, we are aware that it is not widely known, and we therefore present a detailed separate protocol (Precursor technique, at the end of this chapter). We outline two step by step protocols for imaging RNAs live in mammalian cells. Both were used in a prior publication (Braselmann et al., 2018). The first protocol describes mRNA recruitment to stress granules, including quantifying colocalization of mRNA with a stress granule marker protein. Second, we describe a protocol to visualize recruitment of the small, non-coding RNA U1 snRNA to cytosolic U-bodies. Together, these detailed protocols and our efforts to make Cbl-fluorophore probes and plasmid DNA available to researchers should enable a broad community to use Riboglow for diverse RNA imaging applications.

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Figure 1:
Overview of Riboglow platform for fluorescent tagging of RNAs. Top left: The modular Cobalamin (Cbl)-linker-fluorophore probe varies in the organic linker (shaded in blue) and in the fluorophore (shaded in red). Variants that we previously used in cellular studies are shown (three linker variants, two fluorophore variants). Top right: Design considerations of tagging an RNA of interest (drawn in purple) with a variant of the Riboglow RNA tag. Shown are two examples of RNA fusions we previously used in cellular studies (see step by step protocols in this chapter): A<sub>T</sub>-U1 (where one copy of a truncated version of tag variant A, called A<sub>T</sub>, was fused to the 5' end of U1 snRNA) and ACTB-(A)<sub>4x</sub> (where four copies of RNA tag variant A were fused in the 3' untranslated region of the ACTB mRNA). Bottom panel: Upon producing the fusion RNA in cells and introducing the Cbl-fluorophore probe into cells, the RNA tag binds the Cbl portion of the probe and induces light up fluorescence for labeling and tracking.
Figure 2:
Quantification of mRNA localization to stress granules. Recruitment of Riboglow-tagged mRNA to stress granules is quantified by drawing a line trace through the granule (identified in the Halo-G3BP1 channel), and determining the fluorescence ratio in the granule over the cytosolic signal in the Riboglow channel (using background subtracted numbers). Shown are examples from cells where ACTB-(A)4x was labeled with Cbl-4xGly-ATTO 590, and G3BP1 was labeled with JF646 (the full dataset is presented in (Braselmann et al., 2018)). (a) Example of a cell that is suitable for analysis, judged by the observation that stress granules are clearly visible in the Riboglow-ATTO 590 channel, and the Riboglow fluorescence signal in the cytosol is sufficiently above background (signal / background is ~250 / 40 counts). (b) Example of a cell that should be excluded for analysis, because the cytosolic fluorescence signal in the Riboglow-ATTO 590 channel is too close to the background (after
background subtraction, apparent fluorescence counts are <0 and <100 counts). This leads to artificially high ratio values, despite the fact that no apparent recruitment of Riboglow-ATTO 590 to stress granules is visible by inspecting the image. (c) Summary of fluorescence ratio values for a dataset for ACTB-(A)4x labeled with Cbl-4xGly-ATTO 590 versus an untransfected control. To avoid artifacts discussed in (b), cells with cytosolic Riboglow counts <50 after background subtraction were excluded from analysis (this dataset is discussed in detail in (Braselmann et al., 2018)).
Figure 3:
Workflow for imaging ACTB mRNA colocalization with G3BP1-labeled stress granules.
Figure 4:
Workflow for imaging U1 snRNA accumulation in U-bodies.
Figure 5:
Example of results for U-body assay. Left: HeLa cells were transfected with plasmid A₅-U and loaded with 3 μL of 50 μM Cbl-5xPEG-ATTO 590. Cytosolic puncta in the red fluorescence channel form, indicative of U-bodies. Left: Treatment of an untransfected control reveals no cytosolic granules. Scale bar = 5 μm. See (Braselmann et al., 2018) for more examples.
Cbl-fluorophore probes are introduced into live mammalian cells by bead loading, where glass beads are sprinkled onto live mammalian cells to disrupt the mammalian cell membrane and allow entry of small molecules. (a) Schematic to assemble a bead loader. A hole in the center of a cell culture dish is covered with a nylon mesh to allow even distribution of beads onto cells akin to a salt shaker. Care must be taken to contain beads by adding lids on either side to prevent inhalation. (b) Photograph of the assembled bead loader and placement on cell culture dish to sprinkle beads on cells. (c) Workflow to load probes onto cells by bead loading.

**Figure 6:**

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**Table 1:**
Comparison of Riboglow with related techniques

|                         | Riboglow                                               | MS2 system                                           | *In vitro* evolved aptamer-based system (Spinach / Broccoli / Mango) |
|-------------------------|-------------|-----------------------------------------------------|---------------------------------------------------------------|
| **Size of 1x tag**      | 103 nt, 2.3 kDa for fluorophore                       | 61 nt, 88 kDa for fluorophore                        | <100 nt, <0.1 kDa for fluorophore (Broccoli)                  |
| **Fluorogenic probe**   | Cell impermeable probe, mechanical delivery by bead loading. | Genetically encoded protein, must be incorporated in genome or co-transfected. | Cell permeable probe.                                          |
| **Administration option** | Variable; Riboglow-ATTO590 ($\lambda_{ex} = 594$ nm, $\lambda_{em} = 622$ nm) and Riboglow-Cy5 ($\lambda_{ex} = 646$ nm, $\lambda_{em} = 662$ nm) were validated (Braselmann et al., 2018). | Variable; in principle, fluorescent proteins of any color can be fused to the MCP portion. MCP-Halo (e.g., in (B. Wu, Eliscovich, Yoon, & Singer, 2016)) makes synthetic Janelia dyes with variable color choices (Grimm et al., 2017) available. | Invariable; depends on probe for which RNA was evolved ($\lambda_{ex} = 472$ nm, $\lambda_{em} = 507$ nm for Broccoli / DFHBI-1T (Filonov & Jaffrey, 2016), $\lambda_{ex} = 510$ nm, $\lambda_{em} = 535$ nm for Mango / TO1-Biotin (Autour et al., 2018)). |
| **Choice of fluorescent color** | Strong; >60% of cells with stress granules detected via Riboglow-tagged ACTB mRNA (Braselmann et al., 2018). | Strong; >40% of cells with stress granules detected via MS2 SL-tagged ACTB mRNA (Braselmann et al., 2018). | Poor; 10% of cells with stress granules detected via Broccoli-tagged ACTB mRNA (Braselmann et al., 2018). |
| **Photobleaching**      | Relatively photostable due to usage of synthetic fluorophores (Braselmann et al., 2018). | Depends on MCP-fusion; MCP-Halo enables usage of Janelia dyes with favorable photostability properties (Grimm et al., 2017). | Relatively poor in direct comparison of Broccoli and Riboglow (Braselmann et al., 2018), optimization requires engineering of probe and RNA-probe interaction (Li et al., 2019). |
| **Performance in visualizing stress granules via mRNA tag** | Visualization of U1 snRNA demonstrated (Braselmann et al., 2018). | No demonstrated examples due to tag size. | Visualization demonstrated, commonly for Polymerase III-dependent transcripts (highly expressed) (Autour et al., 2018; Song et al., 2017). |
| **Performance in visualizing small non-coding RNAs** | Only demonstrated transient expression from a plasmid under control of strong promoter (Braselmann et al., 2018). | Compatible with endogenous mRNA levels (e.g., (Laonnet et al., 2011)). | Only demonstrated for expression of highly expressed RNAs from a plasmid under control of strong promoter (Autour et al., 2018; Song et al., 2017). |
### Table 2:

Properties of Cbl-fluorophore probes used for live cell imaging

| Probe name       | Absorbance $\lambda_{\text{max}}$ | Extinction coefficient $[\text{L mol}^{-1} \text{ cm}^{-1}]$ | Probe concentration for bead loading | JF dye for orthogonal marker labeling | Fold fluorescence increase upon binding to RNA in vitro (variants A, T, A, B, C, D tested) (measured in (Braselmann et al., 2018)) | Assessment of non-specific localization in live cells (Braselmann et al., 2018) |
|------------------|------------------------------------|---------------------------------------------------------------|-------------------------------------|-------------------------------------|---------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| Cbl-5xPEG-ATTO 590 | 594 nm                             | 120,000                                                       | 3 μL of 50 μM per imaging dish      | JF646                               | 4.0x (bound to A), 4.9x (bound to A), 3.7x (bound to B), 4.0x (bound to C), 2.9x (bound to D) | Diffuse cytosolic localization for 98% of U-2 OS cells / 89% of HeLa cells |
| Cbl-4xGly-ATTO 590 | 594 nm                             | 120,000                                                       | 3 μL of 5 μM per imaging dish       | JF646                               | 7.3x (bound to A), 5.0x (bound to A), 3.9x (bound to D) | Diffuse cytosolic localization for 99% of U-2 OS cells / 98% of HeLa cells |
| Cbl-Cy5          | 646 nm                             | 271,000                                                       | 3 μL of 0.5 μM per imaging dish     | JF585 or SiR594                     | 2.1x (bound to A), 2.7x (bound to A), 2.5x (bound to B), 2.5x (bound to D) | Diffuse cytosolic localization for 98% of U-2 OS cells, localization to non-specific puncta for 63% of HeLa cells |
## Table 3:

### Plasmid information

| Addgene plasmid # | Name                  |
|-------------------|-----------------------|
| 112055            | ACTB-(A)1x            |
| 112056            | ACTB-(A)2x            |
| 112057            | ACTB-(A)3x            |
| 112058            | ACTB-(A)4x            |
| 112059            | AT-U1                 |
|                   | **Available from authors of this chapter** NLS-TagBFP (used as transfection marker; alternatively, any fluorescent reporter protein orthogonal to colors used for Riboglow and G3BP1 labeling can be used) |
Table 4:
Transfection of imaging dishes for stress granule assay

| Dish # | Condition                               | Plasmids transfected      |
|--------|-----------------------------------------|---------------------------|
| 1      | Experimental (transfected, loaded)      | ACTB-(A)4x, NLS-TagBFP    |
| 2      | Control (untransfected, loaded)         | -                         |
| 3      | Control (transfected, not loaded)       | ACTB-(A)4x, NLS-TagBFP    |
| 4      | Control (transfected with marker only, loaded) | NLS-TagBFP                |