Component analysis of nucleolar protein compartments using *Xenopus laevis* oocytes

Emily D. Lavering | Irini N. Petros | Daniel L. Weeks

Department of Biochemistry and Molecular Biology, Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA

Correspondence
Daniel L. Weeks, Department of Biochemistry and Molecular Biology, 4-403 Bowen Science Building, Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA.
Email: daniel-weeks@uiowa.edu

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Abstract
The nucleolus is a multi-compartment, non-membrane-bound organelle within the nucleus. Nucleolar assembly is influenced by proteins capable of phase separation. *Xenopus laevis* oocytes contain hundreds of large nucleoli that provide experimental access for nucleoli that is unavailable in other systems. Here we detail methods to streamline the in vivo analysis of the compartmentalization of nucleolar proteins that are suspected of phase separation. The nucleolus is the main hub of ribosome biogenesis and here we present data supporting the division of proteins into nucleolar domains based on their function in ribosome biogenesis. We also describe the use of vital dyes such as Hoechst 33342 and Thioflavin T in nucleolar staining. Additionally, we quantify nucleolar morphology changes induced by heat shock and actinomycin D treatments. We suggest these approaches will be valuable in a variety of studies that seek to better understand the nucleolus, particularly those regarding phase separation. These approaches may also be instructive for other studies on phase separation, especially in the nucleus.

Keywords
biomolecular condensates, cell nucleolus, fluorescence microscopy, oocytes, *Xenopus laevis*

1 | INTRODUCTION

Distinct compartments within eukaryotic cells allow the efficient organization of many cellular processes. Some of this separation comes from membrane-bound organelles, such as mitochondrion or nuclei. However, a recent paradigm shift in understanding compartmentalization relates to the formation of non-membrane-bound structures mediated by protein condensates and aggregates (Fassler et al., 2021; Hyman et al., 2014; Maji et al., 2009). Some of the critical, early studies on protein condensates were carried out in vitro and established rosters of proteins that would aggregate either by themselves or with aid from a co-aggregate like RNA (Han et al., 2012; Kato et al., 2012). Proteins that can form condensates typically have low complexity and intrinsically disordered regions (IDRs), which are thought to be necessary for protein condensate formation (Alberti et al., 2019; Boeynaems et al., 2019; Feric et al., 2016; Kato et al., 2012; Kato & McKnight, 2017). Condensates may take the form of liquid–liquid phase-separated droplets, gels, and amyloids, all of which have been reported in vitro and in vivo. In vivo studies on protein aggregates have used yeast, nematodes, fruit flies, tissue culture cells, and *Xenopus laevis* oocytes (Banani et al., 2016; Berry et al., 2018; Boke et al., 2016; Brangwynne et al., 2011; Decker & Parker, 2012; Fassler et al., 2021; Feric et al., 2016; Hayes & Weeks, 2016; McSwiggen et al., 2019). Studies that described the phase separation of two nucleolar proteins, nucleoplasmin (Npm1) and fibrillarin (Fbl), provided a visually memorable example of protein...
phase separation within a nucleolus from *X. laevis* oocytes (Feric et al., 2016; Hayes et al., 2018). Here we will discuss our use of *X. laevis* oocytes in studying nucleolar condensates and the advantages of the system.

Historical studies outlined three distinct nucleolar domains using electron microscopy which correspond to sequential steps in ribosome biogenesis. The domains are the fibrillar center where rRNA transcription occurs, the dense fibrillar component where rRNA is modified and processing begins, and the granular component where processing continues and ribosome assembly occurs (Fawcett, 1981; Mais & Scheer, 2001). The nucleolar domains track with the phase separation of sentinel proteins such as Fbl in the dense fibrillar component and Npm1 in the granular component (Feric et al., 2016). However, as important as Npm1 and Fbl are, there are more than 100 proteins in the nucleolus that play a role in ribosome biogenesis and other cellular activities, many of which are suspected of phase separation (Banski et al., 2010; Boisvert et al., 2007; Chen & Huang, 2001; Hayes et al., 2018; Hayes & Weeks, 2016).

We carried out a proteomic study to identify aggregating proteins in the nucleus based on isolation conditions that allow soluble proteins to diffuse away, selectively retaining proteins either in aggregates or stably associated with aggregates (Carroll & Lehman, 1991; Hayes et al., 2018; Paine et al., 1983). Many of these proteins were also identified in studies aimed at characterizing protein aggregates or in studies of the nucleolus (Andersen et al., 2005; Chen & Huang, 2001; Feric et al., 2016; Kar et al., 2011; Kato et al., 2012; Lam & Trinkle-Mulcahy, 2015; Wang et al., 2019).

The approach and data we present here examine the compartmentalization of proteins in the nucleolus. mRNA encoding a protein of interest in frame with a monomeric fluorescent protein was injected into Xenopus oocytes. The ability to direct protein synthesis controlled by injection of mRNA into Xenopus oocytes has been used for over 50 years (Gurdon et al., 1971). The method has been refined over time by dozens of laboratories to produce protein that is synthesized in a living cell, where posttranslational modification, processing, distribution, and interaction with other proteins are all possible. Our particular interest is in the distribution and behavior of phase-separating nucleolar proteins.

## 2 | MATERIALS AND METHODS

### 2.1 | Oocyte removal and preparation

Wild-type *X. laevis* frogs were obtained from Xenopus-1 (Dexter Michigan, USA), Nasco, or from the National Xenopus Resource (at the Marine Biological Laboratory RRID:SCR_013731, Woods Hole, MA). We note that the Nasco Colony is now managed by Xenopus-1. Frogs were anesthetized by immersion in 0.08%–0.1% tricaine (FDA approved, Syndel, USA) in water buffered with NaHCO₃ (pH 7.2) for 15 min. Ovary was aseptically removed and placed in Oocyte Ringers Solution 2 (OR2, 82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄, 5 mM HEPES, and NaOH to pH 7.8). Follicle cells were removed either manually or with collagenase.

### 2.2 | Collagenase treatment

Ovary was sectioned into lobes and placed in 0.2% collagenase (Worthington, type I) in Ca²⁺-free OR2 and rocked at room temperature for 60–90 min. Oocytes were then washed in Ca²⁺-free OR2 supplemented with 0.1% BSA three times followed by five washes with OR2 at room temperature (5 min each). Residual follicle cells were manually removed with watchmaker forceps. Prior to mRNA injection or other treatment, oocytes were left in OR2 at 13°C overnight to recover from collagenase treatment. Oocytes that have follicle cells removed manually can be injected without overnight recovery (Smith et al., 1991). All protocols were approved by the University of Iowa Office of Animal Resources and the Institutional Animal Care and Use Committee.

### 2.3 | Oocyte mRNA injection

mRNA encoding each desired fluorescent fusion protein (10 nl containing 100 ng/µl) was injected directly into each defolliculated oocyte (stage V–VI) using a glass needle paired with a Singer MK-1 (Somerset, England) and an Inject-Matic injector (Geneva, Switzerland). After injection, oocytes were incubated in Oocyte Culture Medium (OCM; 60% Leibovitz’s L-15 medium [containing L-glutamine], 100 µg/ml Pen-Strep, 0.4 mg/ml [all components purchased from Sigma, USA]) at 13°C for at least 24 h prior to microscopy or treatment.

### 2.4 | Fluorescent fusion protein constructs and in vitro synthesis of mRNA

Fbl and Npm1 fluorescent fusion-encoding plasmids (inserted into pCS2+) were gifts from Cliff Brangwynne (Princeton University, Princeton, New Jersey) and were described in Feric et al. (2016). Other fluorescent fusion constructs were made by amplifying a gene of interest (purchased from Transomic Technologies) using NEB’s Q5 PCR and Gibson Assembly Cloning mix (New England Biolabs, E5510S) to insert it in pRN3P (Zernicka-Goetz et al., 1996). The RN3P plasmid was a gift from John Gurdon (Cambridge University, UK). Plasmids were then transformed into *Escherichia coli* DH5α. Some clones with large IDRs required growth in NEB Stable Competent *E. coli* (High Efficiency) (Gar1 and Ncl). See Table S1 for a comprehensive list of proteins, their product numbers, and the primers used to make fluorescent fusions.

The final plasmids have the following features (listed in 5‘→3’ order): either an Sp6 or T3 RNA polymerase promoter, a β-globin 5′ ribosome binding site, our cDNA gene of interest, an in-frame fusion of a fluorescent protein (mGFP5, or RFPs either mKate, mRed, or mCherry [see supplemental table 1]), the 3′ β-globin untranslated region, and a span of 30 A’s and 30 G’s. We note that for studies on protein condensates, monomeric fluorescent proteins help avoid the appearance of aggregation due to the fluorescent protein itself forming multimers (Costantini et al., 2012). We used fluorescent proteins
reported by others to be monomeric. A large database of fluorescent proteins including known aggregation tendencies can be found at https://www.fpbase.org/ (Lambert, 2019). A sample plasmid map can be seen in Figure S1. We tested several of the proteins with the fluorescent protein fusions placed on the N-terminal end and saw similar results. DNA templates for in vitro synthesis of mRNA were made using Q5 polymerase (NEB) directed PCR with either m13F and m13R primers for pRN3P-based clones or M13R and custom primer CATTCTGCTGGGGACGCTC for pCS2+ plasmids. mRNA encoding the fluorescent fusion protein was made using either the SP6 (for Fbl and Npm1) or T3 (for all others) mMessage mMachine in vitro transcription kit following the recommended protocol with lithium chloride RNA extraction (Invitrogen). The concentration of the mRNA was determined using a Nanodrop spectrophotometer (Thermo Scientific) and full-length product was confirmed using gel electrophoresis. mRNA aliquots were stored at −80°C until use.

### 2.5 | Microscopy

Nuclei were manually isolated from oocytes under a dissection microscope in OR2 using watchmaker’s forceps. Representative samples were often prepared using a Zeiss Discovery V8 dissecting microscope with fluorescence. Samples that were analyzed at higher magnification were immediately mounted on a glass microscope slide (in a well with petrolatum sides) and covered with a cover slip. The Zeiss filter sets we used were 1 (Hoechst), 37 (GFP and RNA stains), 43 (RFP), and 47 (ThioT). Images were acquired with an AxioPlan or ApoTome fluorescence microscope running Axio Vision software (RRID:SCR_002677) and an AxioCam Mrm or AxioCam MrC5 camera (Zeiss). The images were acquired using Zeiss’s ApoTome with a 0.25 μm slice size unless otherwise stated. ApoTome images are comparable to confocal microscopy images. Images were processed with Zen 3.3 (blue edition) for further analysis. Images acquired with a 63× objective have 2752 × 2208 pixels for the image size of 198.32 μm × 159.12 μm.

### 2.6 | Image analysis

For each experimental condition, 15 or more nucleoli from each of three nuclei were imaged. Each experiment was repeated at least three times using oocytes from three different frogs. We note that the level of autofluorescence of samples varies from frog to frog. To ensure any fluorescence measured was from fluorescent fusions and not autofluorescence, images were taken of un-injected nuclei at the beginning of each experiment. We typically used fluorescent fusions of either Fbl and Npm1 across all frogs to account for normal variation in size, shape, and number of dense fibrillar components per nucleolus. Differences in size, shape, component proportion, etc., in Figure 1 are all within normal nucleoli variation. We concentrated our studies on nucleoli with multiple dense fibrillar components in each nucleolus.

### 2.7 | Nucleolar staining

Nucleolar DNA was stained with Hoechst 33342 (Thermo Fisher). We stained nucleolar RNA using either Sybr Green II (Thermo Fisher, a gel stain) or Syto Green (Thermo Fisher, a cell stain). We used Thioflavin T (Sigma Aldrich) as an indicator of possible protein amyloid structure. Hoechst (20 μM), Sybr Green II (to 1×), Syto Green (500 nM), or Thioflavin T (50 μM) was added to the OR2, and nuclei were soaked for 1–2 min, rinsed in OR2, and then imaged immediately as described above.

### 2.8 | Heat shock treatment

Oocytes were placed in OCM preheated at 37°C for 1 h (Bienz & Gurdon, 1982; Bouche et al., 1981). Control oocytes were incubated at 13°C (incubation temperature) for 1 h (note: room temperature controls produced the same results). For heat shock recovery experiments, oocytes were placed at 13°C for 1 h after heat shock. Immediately following treatment, nuclei were imaged as described above. For each dataset, color channels in images were split and then the punctate spots, marked by a dense fibrillar component protein, were manually counted in each nucleolus to determine if there was a change in the ratio of punctate spots per nucleolus before and after treatment. Nucleoli that had too many punctate spots to reasonably count were given a value of 20 for statistical purposes. An unpaired t-test of this ratio using GraphPad Prism (RRID:SCR_002798) was used to determine if the change was significant (p < .05).

### 2.9 | Actinomycin D treatment

Oocytes were incubated in 20 μg/ml actinomycin D (Sigma Aldrich) in OR2 for 6 h at room temperature (Mais & Scheer, 2001; Scheer et al., 1975). Afterwards, nuclei were isolated in OR2 and immediately imaged as described above. Images were blinded, and the area of the granular component and dense fibrillar component was determined using ImageJ (RRID:SCR_003070) by first splitting the color channels and then using the “Analyze Particles” function after setting a threshold that was consistent for every image in the dataset. Additionally, the number of dense fibrillar components per nucleolus was counted. RStudio (RRID:SCR_000432) was used to determine if there was a significant difference between the areas and the number of dense fibrillar components per nucleolus before and after treatment using a t-test (p < .05).

### 2.10 | Identification of published proteome data and protein analysis

We found it useful to examine published proteomic data as we planned experiments. For example, we used the supplemental tables in Hayes et al. (2018) as a starting point to identify nucleolar proteins...
with a nuclear pool resistant to rapid diffusion out of the nucleus during isolation in OR2 (Hayes et al., 2018). We also found the whole egg proteome and the nuclear proteomes in the supplemental data published by Wühr et al. (2014) very helpful as well as supplemental protein data in Kato et al. (2012) and Han et al. (2012). There is a growing resource of proteomic data for phase-separating proteins within the entire cell, including the nucleolus. These proteomes are almost always found in supplemental information published with papers of interest. Another resource for proteomic information specific to Xenopus can be found on Xenbase (https://www.xenbase.org, RRID: SCR_003280) under the protein expression tab. There, data accumulated by Peshkin et al. (2019) can be viewed for a variety of Xenopus proteins. We also predicted the IDRs, nuclear localization sequences (NLSs), and nucleolar localization sequences (NoLSs) of each protein of interest using the predictors PONDR-fit, seqNLS, and NoD, respectively (Lin & Hu, 2013; Scott et al., 2010; Xue et al., 2010; RRID:SCR_000997). Visualization of monomer structures used the artificial intelligence-based protein structure predictions in AlphaFold.

**FIGURE 1**  Fluorescent fusion protein nucleolar localization. (a) Schematic of fluorescent fusion protein expression within Xenopus laevis oocyte nucleoli, showing the expression of the canonical domain markers Npm1 (fused with RFP) and Fbl (fused with GFP). (b) Model of the localization of each of these proteins. *Canonical domain markers. (c) Images of representative nucleoli with the labeled fluorescent fusion expressed with either Npm1 or Fbl, the canonical domain markers for the granular component and the dense fibrillar component, respectively. Scale bar = 10 μm.
As of May 11, 2022, over 5000 predicted structures of *X. laevis* proteins have been predicted at [https://alphafold.com/search/text/Xenopus%20laevis?suggested=true](https://alphafold.com/search/text/Xenopus%20laevis?suggested=true).

### RESULTS

We chose proteins for this study from Hayes et al.'s (2018) proteomic study as described above and made a monomeric fluorescent protein fusion for each. A comprehensive list of these proteins, their functions, and information about the presence of a predicted intrinsically disordered region (IDR), nuclear localization sequence (NLS), or nucleolar localization sequence (NoLS) as predicted by Pondr-Fit, seqNLS, and NoD, respectively can be found in Table 1.

| Protein | Function | Citation | Size (kDa) | Presence of IDR/NLS/NoLS (Y = yes, N = no, M = minor (<25 aa disordered)) |
|---------|----------|----------|------------|--------------------------------------------------------------------------------|
| Fbl*    | Member of the rRNA methylation box C/D complex | Iyer-Bierhoff et al. (2018) | 35 | Y | N | N |
| Npm1*   | Ribosome nuclear export | Lindstrom (2011) | 40 | Y | Y | N |
| Gar1    | Member of the rRNA pseudouridination H/ACA complex | Hamma and Ferre-D’Amare (2010) | 22 | Y | N | N |
| Nhp2    | Member of the rRNA pseudouridination H/ACA complex | Hamma and Ferre-D’Amare (2010) | 16 | M | N | Y |
| Snu13   | Member of the rRNA methylation box C/D complex | Watkins and Bohnsack (2012) | 14 | M | N | N |
| Nop56   | Member of the rRNA methylation box C/D complex | van Nues et al. (2011) | 59 | Y | Y | Y |
| Pes1    | Member of the PeBoW complex, involved with cleavage of rRNA | Rohrmoser et al. (2007) | 67 | Y | Y | Y |
| Gtpbp4  | Late maturation of the 60X ribosomal subunit | Fuentes et al. (2007) | 46 | Y | Y | Y |
| Npm3    | Negative regulator of Npm1 | Huang et al. (2005) | 19 | Y | Y | N |
| Ncl     | Multi-functional protein, ribosome assembly and pre-rRNA transcription | Bouvet et al. (1998); Ginisty et al. (1998) | 73 | Y | Y | Y |
| Rpl12   | Large ribosomal subunit | Imami et al. (2018) | 18 | M | N | Y |
| Pak1ip1 | Large ribosomal subunit assembly | Sloan et al. (2013) | 41 | M | Y | Y |

*Canonical domain markers.*

(Jumper et al., 2021). As of May 11, 2022, over 5000 predicted structures of *X. laevis* proteins have been predicted at [https://alphafold.com/search/text/Xenopus%20laevis?suggested=true](https://alphafold.com/search/text/Xenopus%20laevis?suggested=true).

### FIGURE 2

Representative images of Hoechst 33342, Sybr Green II, Syto Green, and Thioflavin T nucleolar staining as indicated by the label on each image. The Hoechst images are whole field fluorescence images, while all other images are apotome section fluorescence images. Scale bar = 10 μm
be incubated at 18°C for accelerated protein expression, with a shorter survival period.

Nuclei were isolated from the oocytes in OR2. Unfixed nuclei were then imaged using ApoTome fluorescence microscopy. The contents and activities within each domain have previously been proposed to represent the sequential steps of ribosome biogenesis, where proteins involved in early, intermediate, and late stages localize to the fibrillar center, dense fibrillar component, and granular component, respectively (Figure 1b). Figure 1c shows representative nucleoli containing fluorescent fusion proteins, including examples of dense fibrillar component- and granular component-localizing proteins. The proteins that co-localized with Fbl in the dense fibrillar component include those involved in rRNA modification, such as Snu13, Gar1, Nhp2, and Nop56. Proteins involved in ribosome assembly, maturation, and export, such as Gtpbp4, Npm3, Ncl, and Pes1, co-localize with Npm1 in the granular component. We note that the crisp phase separation seen when comparing Fbl and Npm1 as markers of the dense fibrillar component and the granular component, respectively, extends to some but not all the nucleolar proteins. For example, while Nop56 primarily localizes to the dense fibrillar component, it also partially partitions into the granular component (Figure 1c). Dense fibrillar component localization was anticipated, as Nop56 is part of the same rRNA methylation complex as the canonical dense fibrillar component domain marker Fbl. New models of the nucleolus will need to account for proteins that drift between compartments. While the proteins that join the dense fibrillar component seem homogeneously distributed at the resolution obtained, the granular component protein localization was not as evenly overlapping (see Npm1 and Pes1 image from Figure 1c). These findings indicate that a more nuanced model may need to include additional sub-compartments.

In addition to fluorescent protein fusions studies, DNA or RNA within the nucleolus can be visualized using fluorescent stains that diffuse into the nucleus. Nuclei were isolated in OR2, and then stains were added to the solution. Hoechst 33342 stains DNA, including the extra-chromosomal rDNA in the fibrillar center of nucleoli. This makes it an easily accessible marker for the fibrillar center (Figure 2). We have shown previously that Sybr Green II stains nucleoli (Hayes & Weeks, 2016), and here we show that Syto Green can also be used to stain RNA within the nucleolus. Both primarily stain the granular component (Figure 2). Syto Green was slightly brighter than Sybr Green II at the recommended concentrations.

Thioflavin T is a probe that was developed to identify amyloid structures in neurodegenerative diseases such as Alzheimer’s disease and we have detailed its use in oocytes previously (Hayes & Weeks, 2016; Vassar & Culling, 1959). Thioflavin T is a small molecule that fluoresces when bound to amyloid structures (Vassar & Culling, 1959). In solution, Thioflavin T molecules have free rotation around a central carbon bond that is restricted when bound to

![Image](image-url)
amyloid structures, which releases energy as fluorescence. While Thioflavin T is commonly used to detect amyloid structures, some ordered RNA structures can also be stained by it (Xu et al., 2016). In vitro phase separation studies have shown that RNA has the ability to increase the order of protein condensates (Feric et al., 2016; Kato et al., 2012). Whether Thioflavin T is indicating amyloid structures or ordered RNA, it does stain the nucleolus, primarily the granular component.

The nucleolus is dynamic and must be able to respond to various stresses. Here we show how morphological changes after heat shock and actinomycin D treatment can be assessed (Figures 3–5). Heat shock invokes an overall stress response. As part of this response, ribosomal RNA processing is compromised and total rRNA accumulation is reduced (Labhart & Reeder, 1987). Actinomycin D inhibits RNA polymerase I, which stops the production of new rRNA in stage V–VI oocytes (Roger et al., 2002). We compared nucleolar morphology changes after heat shock and actinomycin D treatment, as both compromise ribosome biogenesis. While we expected the morphological changes induced by the two treatments to be similar, we found that they cause almost opposite phenotypes.
Oocytes were heat shocked at 37°C for 1 h. To determine optimal heat shock treatment, we tested heat shock durations from 20 min to 2 h. We found that the greatest morphological changes occur after 1 h and the difference between 1 and 2 h was negligible. Optimization of temperature was done previously (Bienz & Gurdon, 1982; Labhart & Reeder, 1987). We also found that oocytes ruptured when heat shocked at temperatures higher than 37°C. After heat shock, nuclei were isolated and nucleoli were imaged (Figures 3a and 4a). We manually counted the number of punctate spots in the widest apotome slice of nucleoli comparing heat shocked versus control oocytes (Figures 3b and 4b). We counted the number of punctate spots per nucleolus and found that the dense fibrillar components breaks apart and begin to diffuse into the granular component after heat shock (Figures 3 and 4). Heat shock morphology can be reversed by incubation at 13°C. As shown in Figure 3, Fbl and Npm1 are restored to their positions in the dense fibrillar component and granular component, respectively, after recovery. Other proteins that localize to the dense fibrillar component were tested and mimic the pattern shown for Fbl in response to heat shock (Figure 4).

In contrast to heat shock, incubating oocytes in 20 μg/ml actinomycin D for 6 h increases the area of and decreases the number of dense fibrillar components per nucleolus (Figure 5a,b). Additionally, the area occupied by the granular component, as marked by Npm1, decreases upon actinomycin D treatment. Other proteins that localize to the dense fibrillar component or the granular component follow the same trend after treatment, as they co-localize with Fbl and Npm1, respectively (Figure 5c). These results mirrored earlier studies in HeLa cells that were observed via electron microscopy (Reynolds et al., 1964); however, X. laevis oocyte nuclei provide a system where unfixed samples are observed and isolation and treatment conditions can be easily modified. Previous studies have suggested that dense fibrillar component condensing is due to the collapse of the inactive chromosomal rDNA pulling the tethered proteins of the dense fibrillar components together (Tchelidze et al., 2017). Xenopus laevis oocytes have hundreds of nucleoli that surround circular, extra-chromosomal rDNA, yet the collapse of the dense fibrillar component after actinomycin D treatment still occurs. It is important to note that actinomycin D treatment 72 h or more post-mRNA injection did not always produce statistically significant differences in the ratio of dense fibrillar components per nucleolus. This was because as the oocytes age, the control groups in some experiments had a lower number of dense fibrillar components per nucleolus. We recommend actinomycin D treatment experiments be carried out 24–48 h post-mRNA injection. We saw the reversal of the morphology changes caused by heat shock treatment after a period of recovery; however, we were unable to reverse the changes induced by actinomycin D treatment.

4 | DISCUSSION

4.1 | Advantages and limitations of this method

There are many advantages of using X. laevis oocytes to study multi-protein nucleolar phase separation, a major advantage being size. Stage VI Xenopus oocytes and their nuclei have diameters of ~1.3 mm and 400 μm, respectively (Brangwynne et al., 2011; Dumont, 1972;
4.2 | Expertise required to implement the method and helpful Xenopus tools

To carry out these studies, animal training specific to X. laevis is required. Expertise in fluorescence microscopy and proficiency in standard molecular biology techniques (for synthesis of fluorescent fusion clones, synthesis of mRNA, and troubleshooting) are also needed to implement these methods.

Xenbase (https://www.xenbase.org, RRID:SCR_003280) is an invaluable tool for any Xenopus research. On Xenbase information is available about the expression, phenotype, isoforms, interactomes, antibodies for, and literature related to a specific protein of interest. Additionally, Xenbase has a wealth of protocols and information on reagents specifically for Xenopus research. We utilized numerous Xenopus-specific protocols from Cold Spring Harbor (http://cshprotocols.cshlp.org/cgi/collection/xenopus). The books The Early Development of Xenopus Laevis: An Atlas of the Histology and Xenopus laevis: Practical uses in cell and molecular biology were also instrumental for reagents, protocols, and understanding the development of X. laevis (Hausen & Riebesell, 1991; Kay & Peng, 1991).

4.3 | Importance of new findings and applications of the method

The nucleolus is the main hub for ribosome biogenesis, which is a vital process within the cell. Our understanding of the role protein aggregation plays in ribosome biogenesis has primarily been based on canonical domain markers, yet nucleolar condensates contain many proteins. Here we show the localization of numerous nucleolar proteins that are suspected of phase separation, including Gar1, Nhp2, Snu13, Nop56, Pes1, Ncl, Npm3, Gtpbp4, Rpl12, and Pak1ip1. From these studies we created a more nuanced model of the nucleolus.

The nucleolus is dynamic and must be able to respond to various types of stress. It is affected by various cell states, including cell division, aging, and various diseases. Additionally, outside of ribosome biogenesis, the nucleolus is a site where both useful and toxic aggregating proteins are regulated (Frottin et al., 2019). Xenopus laevis oocytes are a useful tool to gain a clearer picture of the nucleolus in various stress and disease states. Here we show the drastic difference in response elicited by two conditions known to decrease ribosome biogenesis, heat shock and actinomycin D treatment. Furthermore, we were able to examine these changes using various nucleolar proteins.

This system can also be used to assess how biologically relevant mutations in nucleolar proteins affect localization to or the structure of the nucleolus under various conditions. Many of the proteins examined here are members of multi-protein complexes and we are currently examining the relationship between complex formation and phase separation. These studies are instructive for other multi-compartment, non-membrane-bound organelles. Many of these techniques could be directly applied to other nuclear non-membrane-bound organelles.

AUTHOR CONTRIBUTIONS

EDL and DLW conceived and designed the project. EDL and INP acquired and analyzed the data. DLW did all microinjection. EDL and DLW wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.
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