Journal of Visualized Experiments

Production of Xenopus tropicalis egg extracts to identify microtubule-associated RNAs.

--Manuscript Draft--

Manuscript Number:	JoVE50434R4
Article Type:	Invited Methods Article - JoVE Produced Video
Corresponding Author:	Mike Blower, PhD Massachusetts General Hospital Boston, MA UNITED STATES
First Author:	Judith Sharp
Order of Authors:	Judith Sharp
	Mike Blower, PhD

Cover Letter

MASSACHUSETTS GENERAL HOSPITAL

HARVARD MEDICAL SCHOOL

Michael D. Blower, Ph.D.
Assistant Professor of Genetics
Harvard Medical School

Department of Molecular Biology
185 Cambridge St, CPZN-7250
Boston, MA 02114
blower@molbio.mgh.harvard.edu

Dear Dr. Chen,

We are submitting our article entitled "Production of *Xenopus tropicalis* egg extracts to identify microtubule-associated RNAs" for publication in JoVE. In this work we describe a method for producing meiosis-II arrested egg extracts from the frog *X. tropicalis.* We then describe a simple method to purify microtubules from this extract to identify RNAs that are specifically bound to microtubules. We think that this method will be of general interest to groups working on RNA localization as it describes a novel method for identifying localized RNAs. We also think that this method will be generally interesting to the *Xenopus* community because it describes a method to make egg extracts from a frog species with a sequenced genome, *X. tropicalis*, as opposed to the much more commonly used *X. laevis*, which does not have a sequenced genome. In our discussion we suggest several possible areas for improvement of this method and ways that it can be generalized to other cellular organelles.

We hope that you find this work suitable for publication in JoVE.

Sincerely,

Mike Blower

Production of *Xenopus tropicalis* egg extracts to identify microtubule-associated RNAs.

Judith A. Sharp[1,2] and Michael D. Blower[1,2,3]

[1] Department of Molecular Biology, Massachusetts General Hopsital. Boston, MA 02114
[2] Department of Genetics, Harvard Medical School. Boston MA 02115
[3] To whom correspondence should be addressed: blower@molbio.mgh.harvard.edu

Keywords: *Xenopus* , microtubules, egg extract, RNA localization

Short Abstract

We describe the collection of unfertilized *Xenopus tropicalis* eggs and production of a meiosis II-arrested egg extract. This egg extract can be used to purify microtubules and microtubule-associated RNAs.

Long Abstract

Many organisms localize mRNAs to specific subcellular destinations to spatially and temporally control gene expression. Recent studies have demonstrated that the majority of the transcriptome is localized to a nonrandom position in cells and embryos. One approach to identify localized mRNAs is to biochemically purify a cellular structure of interest and to identify all associated transcripts. Using recently developed high-throughput sequencing technologies it is now straightforward to identify all RNAs associated with a subcellular structure. To facilitate transcript identification it is necessary to work with an organism with a fully sequenced genome. One attractive system for the biochemical purification of subcellular structures are egg extracts produced from the frog *Xenopus laevis.* However, *X. laevis* currently does not have a fully sequenced genome, which hampers transcript identification. In this article we describe a method to produce egg extracts from a related frog, *X. tropicalis,* that has a fully sequenced genome. We provide details for microtubule polymerization, purification and transcript isolation. While this article describes a specific method for identification of microtubule-associated transcripts, we believe that it will be easily applied to other subcellular structures and will provide a powerful method for identification of localized RNAs.

Introduction

Spatial and temporal control of gene expression is important for all cells, and is especially important for the control of early embryonic pattering[1]. Spatial control of gene expression is achieved through the active localization of mRNAs to specific destinations within cells or embryos. In many very large cell types, (e.g. oocytes, embryos, and neurons) mRNA localization is used to restrict protein expression to the site of action of the coded protein. Since a localized mRNA can catalyze many rounds of protein production it is more efficient to localize an mRNA than to localize individual protein molecules. Localized mRNAs are typically translationally repressed until they reach their destination, which serves to further limit the localization of the coded protein[2]. In addition to the many well-documented cases of RNA localization to control embryonic patterning, several studies have documented mRNAs that are localized to the site of action of the encoded protein. Prominent examples include localization of the β-actin[3] and Arp2/3[4] mRNAs to the leading edge of motile fibroblasts and localization of the mRNAs for many mitotic regulators to meiotic and mitotic spindles[5-7].

Many of the classic examples of localized mRNAs were identified through genetic screens for maternal effect mutations and were later determined to encode localized RNAs. However, recent genome-wide studies have begun to provide broader insight into the scope of localized RNAs. A recent *in situ* hybridization screen in *Drosophila* embryos demonstrated that ~70% of all mRNAs have a specific localization, including many novel destinations[8]. Purification of pseudopodia from mouse fibroblasts identified a diverse group of localized mRNAs[9]. Work from our group using biochemical purification of microtubules from meiotic *Xenopus* egg extracts identified hundreds of mRNAs that copurify with the spindle[5,7]. Our work showed that the majority of microtubule-localized mRNAs encode proteins that function in control of mitosis, supporting the idea that mRNAs are localized to the site of action of the coded protein. Furthermore, the ability to detect mRNA enrichment in a subcellular fraction by biochemical purification highlights the power of this approach for identification of localized mRNAs.

Most localized RNAs use active transport on the cytoskeleton, either actin or microtubules, to achieve transport to their final destination[10]. To gain a better understanding of the extent and types of RNAs that are localized to specific destinations using a biochemical approach it is necessary to have an *in vitro* system that can recapitulate cytoskeletal processes. One of the premier systems for studying cytoskeletal biology is egg extracts produced from unfertilized eggs from the frog *Xenopus laevis*. *X. laevis* egg extracts have been used for decades to study a wide array of cytoskeletal processes and have contributed much to our understanding of the mechanisms and molecules that control cytoskeletal assembly and dynamics[11]. Furthermore, *X. laevis* egg extracts are amenable to large-scale purifications of microtubules and associated proteins[12,13] and there are well-designed methods for the production of various types of egg extracts[14-16]. However, for genomic studies there are several drawbacks to the use of *X. laevis* as a model system.

For decades *Xenopus laevis* frogs have been a powerful system for the study of developmental and cell biology, owing to the large oocyte size and robust external development[17]. Furthermore, the development of egg extract systems that can

recapitulate many cellular processes in a test tube has made this frog a powerful experimental model. However, *Xenopus laevis* has been hampered by the lack of a complete genome sequence, which has been slowed by the allotetraploid nature of the genome. In contrast, a closely related species, *Xenopus tropicalis,* has a diploid genome that was sequenced in 2010[18]. While *X. tropicalis* is not as experimentally tractable as *X. laevis*[17] the availability of a sequenced genome makes it an attractive model system to perform genome wide analyses.

In this report we describe a method to make meiosis II-, cytostatic factor-arrested extracts (CSF) from *X. tropicalis*[19]*.* We then describe a simple method to purify microtubules and associated RNAs from this extract. The RNAs can then be converted into libraries amenable to sequencing using recently developed high throughput sequencing technologies. Once the libraries are sequenced they can be aligned to the genome of the frog to identify specific mRNAs that are enriched in the microtubule sample compared to total extract. This provides a powerful method to detect microtubule-targeted mRNA localization on a genome-wide scale. In addition to being able to detect localized mRNAs, the use of high-throughput sequencing and a sequenced genome offer the possibility of discovering novel transcripts that are not currently present in public database annotations.

PROTOCOL:

1) Generation of *X. tropicalis* eggs

All *Xenopus tropicalis* frogs are ordered from NASCO. Our frogs are housed in an Aquatic Habitats recirculating water system kept at 27°C. There are many options for water systems for care of *X. tropicalis.* Some good general information on this frog species can be found on the web sites of the Harland and Grainger labs (http://tropicalis.berkeley.edu/home/, http://www.faculty.virginia.edu/xtropicalis/). Our frogs are maintained in tankwater consisting of (0.4g Ciclid Lake Salts, 0.6g marine salt, 0.625g$NaHCO_3$ per liter of water, pH 7.0)[20]. This recipe results in a conductivity of ~1800 µS, which is a high salinity for *X. tropicalis.* However, we have found that our frogs thrive in this environment and oocyte quality is improved. Alternative tankwater recipes can be found above at the resources listed for general *X. tropicalis* care.

1.1) Frogs are injected with human Chorionic Gonadotropin (hCG) on three successive days to stimulate egg laying: First, prepare two concentrations of hCG solution. Resuspend 10,000 U of lyophilized hCG powder in 10 mL sterile, deionized H_2O for a final concentration of 1000 U/mL. Then, dilute 1 mL of 1000 U/mL hCG solution in 9 mL H_2O for a final concentration of 100 U/mL. Store both solutions at 4°C.

1.2) On day 1, prepare 4-6 frogs for egg laying by injecting with hCG between 2:00-3:00 PM. Inject each frog in the dorsal lymph sac near the cloaca with 0.2 mL 100 U/mL hCG solution. Having the frogs fast during the subsequent two injections will minimize the amount of frog waste present during egg laying, but is optional.

1.3) On day 2, inject the same frogs with 0.2 mL 100 U/mL hCG solution between 2:00-3:00 PM.

1.4) On day 3, inject the same frogs with 0.2 mL 1000 U/mL hCG solution, between 7:00-10:00 AM. Set up frogs to lay eggs: fill a 6-quart plastic bucket with fresh tankwater, add frogs and place in the dark at 25°C. After this injection, egg laying will begin after 4 hours and will be complete by 7 hours. Frogs should lay eggs in an environment that is maintained at a minimum of 25°C.

1.5) Make extract solutions and have equipment ready immediately before collecting eggs.
20X MMR: 100 mM HEPES, pH 7.8; 2 mM EDTA pH 7.8; 2 M NaCl; 40 mM KCl; 20 mM $MgCl_2$; 40 mM $CaCl_2$. Autoclave and store at room temperature. Prepare 1 L of 1X MMR just prior to extract preparation.
10X XB: 100 mM HEPES, pH7.7; 10 mM $MgCl_2$; 1 mM $CaCl_2$; 1 M KCl; 500 mM sucrose. Autoclave and store at 4°C. Prepare 1 L of 1X XB just prior to extract preparation.
Dejelly solution: Prepare 250 mL 3% cysteine solution in deionized H_2O and pH to 7.8-8.0 with 10 N NaOH. Prepare just prior to extract preparation.
CSF-XB: take 200 mL of 1X XB and add 2 mL 0.5M EGTA pH 7.7 and 200 µL 1 M $MgCl_2$. Prepare just prior to extract preparation.
CSF-XB+: take 50 mL of CSF-XB and add 50 µL of LPC (10 mg/mL each stock of Leupeptin, Pepstatin, and Chymostatin in DMSO). Add 50 µL Cytochalasin D (10 mg/mL in DMSO). Prepare just prior to extract preparation.
Prepare a 0.2% gelatin solution in deionized H_2O, microwave to dissolve and filter sterilize. Store at room temperature.
Reserve 2 Beckman 2 x ½ inch Ultracentrifuge tubes.
Prepare two 15 mL glass round-bottomed centrifuge tubes with 0.5 mL of H_2O in each to cushion the ultracentrifuge tube.

Make fire-polished glass Pasteur pipets. Snap the end off of 5 ¾ inch glass pipets to expose a broad opening, and expose to flame to smooth the new exposed pipet tip.

1.6) Prepare a 500 mL glass beaker for storing eggs by swirling a 0.2% gelatin solution around to coat the walls of the beaker. Discard gelatin solution from beaker after use.

1.7) Collect eggs from the plastic bucket used for laying 6-7 hours after the third injection on day 3. If desired, gently squeeze each frog once to get any remaining eggs. Wash eggs once with fresh tankwater and transfer to the 500 mL glass beaker coated with 0.2% gelatin solution.

2) Preparation of extract from *X. tropicalis* eggs
All steps of extract preparation can be performed at room temperature, approximately 25°C. Throughout the washes, it is important to keep the eggs submerged under liquid

so that they remain wet. Exposure to air can cause the eggs to escape cell cycle arrest or lyse.

2.1) Decant as much tankwater as possible while reserving enough liquid to keep the eggs wet. Tilt the beaker containing eggs to the side and add ~300 mL 1X MMR slowly to the wall of the beaker, so that physical agitation of the eggs is minimized. Let eggs settle, then decant off supernatant containing debris. *X. tropicalis* eggs are stringy at this step, so removal of activated eggs is done after dejellying. Repeat for a total of three 1X MMR washes.

2.2) Dejelly the eggs. Decant off as much MMR as possible and add half of the dejelly solution. Swirl continuously for approximately 5 minutes. Dissolving jelly coats will be visible in the supernatant after a couple of minutes. Decant off and add the remaining dejelly solution. Continue to swirl continuously until eggs pack very tightly and all orient with their vegetal pole (the pole with white pigment) toward the bottom of the dish. Quickly decant off as much dejelly solution as possible. Once the eggs are dejellied they are very sensitive to mechanical manipulations.

2.3) Carefully add XB to the eggs. In the first XB wash, remove eggs that have escaped CSF arrest by removing lysed, puffy, white, and pseudocleavage eggs. Activated *X. tropicalis* eggs tend to settle in the top center, so use a plastic transfer pipet to pull these out. Also remove pieces of skin and frog waste. Wash eggs a total of three times with ~ 300 mL 1X XB solution, gently swirling eggs between washes and allowing them to settle on the bottom of the beaker. As before, decant as much of each wash solution as possible while keeping eggs wet.

2.4) Wash eggs twice with CSF-XB and decant.

2.5) Add CSF-XB+ to eggs. Using a gelatin-treated fire-polished Pasteur pipette, transfer eggs to Ultra-centrifuge tubes with CSF-XB+, taking care not to expose the eggs to air. Place inside the 15 mL glass centrifuge tubes with the water cushion.

2.6) Spin eggs in a clinical centrifuge at 200 x g for 1 minute, increase speed to 800 x g and spin for 30 seconds.

2.7) Use an aspirator to remove as much buffer as possible from eggs. They should be almost dry on top. Quickly move eggs to a Sorvall RC-6 centrifuge equipped with a HB-6 rotor (or equivalent) and spin 17,000 x g for 15 minutes at 20°C.

2.8) Remove the yellow cytoplasmic layer between the pigment and lipid layers using an 18 gauge needle attached to a 1 mL syringe. Puncture the side of the tube and pull the syringe barrel slowly to obtain the cytoplasmic extract layer. Avoid pigment granules as much as possible.

2.9) Transfer cytoplasm to new ultracentrifugation tube. It is normal for the extract to appear slightly cloudy at this step. Place inside the 15 mL glass centrifuge tube with water cushion. Spin again 17,000 x g for 10 minutes at 20°C. Repeat extraction with 18 gauge needle.

2.10) Transfer cytoplasm to a 1.5 mL microfuge tube. Estimate the extract volume and dilute Cytochalasin D and LPC 1:1000 into the extract. Mix well with a 1 mL pipet tip, pipetting up and down many times without introduction of air bubbles. A typical yield from a healthy frog colony is approximately 300-500 μL of extract/frog. To preserve maximum activity, it is necessary to store the extract and perform experimental manipulations at room temperature (20-25°C).

3) Purify taxol-stabilized microtubules from *X. tropicalis* extract

3.1) Add Taxol to a 100-200 μL aliquot of extract at a final concentration of 10 μM and incubate at room temperature for 30 minutes. For control reactions, treat an equivalent volume of extract with the microtubule-destablilzing drug Nocodazole (10 μM). Reserve 100 μL of untreated extract for analysis.

3.2) Dilute the drug-treated extract with 10 volumes BRB-80 (80 mM PIPES pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) + 30% glycerol. Assemble 14 mL round-bottom polypropylene tubes containing 10 mL of BRB-80 + 60% glycerol cushion. Using a wide bore pipet tip, layer the drug-treated extract reaction gently on top of the BRB-80 + 60% glycerol cushion. Centrifuge for 10 minutes at 17,000 x g at 20°C in a Sorvall RC-6 centrifuge equipped with a HB-6 rotor (or equivalent) and tube adapters.

3.3) Aspirate the supernatant containing unsedimented extract material, and wash the interface twice with deionized H_2O. Aspirate the remaining cushion volume slowly, taking care not to disturb the gel-like pellet containing microtubules, microtubule-associated proteins, and microtubule-associated RNAs in the Taxol-treated sample. The Nocodazole-treated sample does not contain visible material. Resuspend the pellet in 1 mL TRIzol and proceed with the manufacturer's instructions for isolating RNA. Untreated extract (up to 100 μL) can be resuspended directly in 1 mL TRIzol.

3.4) There are now commercially available kits for preparing transcriptome libraries suitable for RNA-seq. These may be purchased through http://www.illumina.com/ and http://www.454.com/.

REAGENTS:

Name	Company	Catalogue Number
Xenopus tropicalis	NASCO	LM00823MX
human Chorionic Gonadotropin	Sigma-Aldrich	CG10

HEPES	Sigma-Aldrich	H4034
EDTA	Sigma-Aldrich	E5134
NaCl	Sigma-Aldrich	S3014
KCl	Sigma-Aldrich	P9541
$MgCl_2$	Sigma-Aldrich	M8266
$CaCl_2$	Sigma-Aldrich	C8106
sucrose	Sigma-Aldrich	S0389
NaOH	Sigma-Aldrich	S5881
EGTA	Sigma-Aldrich	E3889
Leupeptin	Sigma-Aldrich	L9783
Pepstatin	Sigma-Aldrich	P5318
Chymostatin	Sigma-Aldrich	C7268
Cytochalasin D	Sigma-Aldrich	C8273
Gelatin, porcine skin	Sigma-Aldrich	G1890
PIPES	Sigma-Aldrich	P6757
Taxol	Sigma-Aldrich	T7191
Nocodazole	Sigma-Aldrich	M1404
Trizol	Invitrogen	15596-026
L-Cysteine, free base	USB Corporation	14030
Cichlid Lake Salt	Seachem	47894
Marine salt	Seachem	SC7111
$NaHCO_3$	Sigma-Aldrich	S6014

EQUIPMENT:

Material Name	**Company**	**Catalogue Number**
1 mL syringes	BD Biosciences	309659
18 gauge needles	BD Biosciences	305195
30 gauge needles	BD Biosciences	305106
Rubbermaid Plastic bucket	Amazon	6306
Beckman Polyallomer 2 x ½ inch Ultracentrifuge tubes	Beckman	326819
15 mL round-bottomed glass centrifuge tubes	Fisher Scientific	45500-15
Rubber adapter sleeves for 15 mL tubes	Kimble-Chase	45550-15
5 ¾ inch glass Pasteur pipets	Fisher Scientific	13-678-20A
14 mL polypropylene round-bottom tube	BD Biosciences	352059
Sorvall HB-6 rotor	Thermo Scientific	11860
Sorvall RC-6 centrifuge	Thermo Scientific	46910

REPRESENTATIVE RESULTS:

To identify *X. tropicalis* transcripts associated with microtubules, we prepare a cytosolic extract from unfertilized eggs arrested in metaphase of meiosis II (CSF). Treatment of this extract with taxol allows the formation of stable microtubules that can be purified by sedimentation through a glycerol cushion (Figure 1A). Coomassie gel analysis confirms that α/β-tubulin sediments in a taxol-dependent manner, and represents the major protein species recovered in these preparations (Figure 1B). Lower levels of other proteins are also present in the taxol pellet, but not in preparations treated with the microtubule depolymerizing drug nocodazole, indicating that proteins in the taxol fraction specifically associate with microtubules (MAPs).

An Agilent Bioanalyzer is used to examine general RNA composition in all *X. tropicalis* extract fractions (Figure 1C). Both rRNA and tRNA species are present in CSF extract and the microtubule-containing taxol pellet, consistent with previous findings that translation occurs on microtubules and spindles in *X. laevis* egg extract[5,21]. A line trace of the gel projection reveals the mRNA signal is markedly lower in the microtubule-containing taxol pellet, most notably in the region migrating above 28S rRNA, indicating that a subset of mRNAs cosediment with microtubules in *X. tropicalis*. RNA isolated in this manner is suitable for RNA-seq experiments using commercially available reagents.

FIGURE LEGEND:

Figure 1. Purification of MT-RNA for RNA-seq. (A) Purification scheme to isolate MT-RNA. Eggs are harvested from female *X. tropicalis* frogs. After preparation of a cytoplasmic extract, taxol is added to induce microtubule polymerization. Microtubules and MT-RNA are purified by sedimentation through a glycerol cushion. (B) Coomassie gel analysis of proteins isolated using the scheme described in (A). Total CSF extract compared to proteins sedimented in the presence of taxol or nocodazole. (C) Bioanalyzer gel analysis of RNA isolated using the scheme described in (A). RNA isolated from CSF extract compared to RNA sedimented in the presence of taxol or nocodazole. Both the gel projection and the line traces are shown. Reprinted with permission from Sharp et al., 2011.

Discussion

In this report we have described a simple method to produce CSF-arrested egg extracts from *X. tropicalis*[19] and use this extract to study microtubule-associated RNAs[7]. The basic procedure for producing CSF-arrested egg extracts from *X. tropicalis* is the same as used for *X. laevis* with a few key differences. One of the most challenging aspects to working with *X. tropicalis* frogs is obtaining enough high quality eggs to make an extract with microtubule nucleation or spindle assembly activity comparable to *X. laevis* egg extracts. To achieve optimal egg laying conditions while preventing slippage

from meiosis II cell cycle arrest, the interval between hormone injections for *X. tropicalis* is shorter than that used for *X. laevis,* and the timing from the third hCG injection to the beginning of egg laying is also much shorter. With *X. laevis* the timing from the hCG injection to the beginning of egg laying is such that it is convenient and efficient for eggs to be laid overnight into buffer. However, because of the shorter time between hCG injection and egg laying with *X. tropicalis* it is frequently necessary to manually express the eggs from frogs. Another significant difference between making egg extract from the two different frogs is the dejellying step. With *X. laevis* the eggs are so large that it is easy to determine when the jelly coat has dissolved by observing how closely the eggs are spaced in the beaker. As the dejellying reaction commences, the eggs begin to pack more densely. However, *X. tropicalis* eggs are much smaller and it can be quite difficult to determine when the jelly coat has dissolved by egg packing density alone. We have found that the most reliable method to determine when the jelly coat has dissolved is to monitor the orientation of the animal (black) and vegetal (white) poles. When all the vegetal poles orient toward the bottom of the beaker the jelly coat has been removed enough to proceed with the extract. Finally, whereas *X. laevis* egg extract can be stored at cool temperatures (4-12^{o}C) we have observed that it is critical to maintain *X. tropicalis* egg extract at room temperature (20-25^{o}C) during preparation and experimental manipulations to preserve biochemical activity. Because of the differences in ease of use we prefer to use *X. laevis* frogs for the production of egg extract. However, for experiments that require or are facilitated by an organism with a sequenced genome, *X. tropicalis* is an excellent alternative system.

The method that we have described in this report uses taxol as a microtubule-stabilizing agent to induce microtubule polymerization. We chose this method because taxol is a robust microtubule-stabilizing agent that facilitates the large-scale isolation of purified microtubules. The method that we described could likely be improved by comparing the proteins and RNAs associated with microtubules using alternative microtubule polymerization methods. Alternatives could include polymerization using GTP-induced polymerization (a classic technique),[22] or using Ran-GTP as a microtubule polymerizer to mimic the microtubules induced by chromatin-driven spindle assembly[23]. Finally, use of purified sperm nuclei to induce microtubule polymerization would be the closest mimic to the types of microtubules that are nucleated during mitosis (centrosome, chromatin, and kinetochore mediated). Drawbacks to these alternative sources of microtubule nucleation are that the nucleating agents are not as readily available as taxol and they do not nucleate or stabilize microtubules as efficiently as taxol. Therefore, each of these methods would be more difficult to use for large-scale purifications. The advantage of comparing multiple different types of microtubule nucleators is that it could be possible to identify proteins and/or RNAs that are specific to each pathway of microtubule nucleation.

The method that we have described here takes advantage of cytoplasmic extracts of amphibians. However, this approach could be extended to the use of extract system from other organisms. Mitotic extracts have been described from synchronized human tissue culture cells[24] that faithfully recapitulate many aspects of microtubule assembly. We have successfully used these extracts to identify microtubule-associated

RNAs from HeLa cells[5]. Similar microtubule purification schemes have been described for many different organisms[25,26], although the microtubule associated RNAs have not been examined. The approach described here could be used with any organism that can produce a concentrated cytoplasmic extract capable of nucleating microtubules.

Finally, although the approach that we describe here discusses the purification of microtubules and associated proteins and RNAs, this approach could be generalized to other subcellular structures. While most localized mRNAs have not been identified using biochemical methods the recent advances in DNA and RNA sequencing technologies make this approach an attractive method to identify localized RNAs. In this approach any subcellular or sub-embryo structure of interest could be isolated or purified. Then the associated proteins and RNAs can be identified on a genome wide scale. RNAs can then be compared to the RNA content of the total cell or embryo to identify enriched localized RNAs. This approach could be used with whole eggs (animal and vegetal separation, similar to the approach that identified the first localized RNAs in *Xenopus*[27]*),* actin associated RNAs, ER-associated RNAs, mitochondria-associated RNAs, or to any subcellular structure that can be purified with associated RNAs intact. Based on our work on microtubule-associated RNA we predict that this would be an excellent method to discover new proteins that function at a given location. Furthermore, identification of the location and extent of all localized RNAs will provide insight into how cells and embryos use mRNA localization to control gene expression.

References

1 Martin, K. C. & Ephrussi, A. mRNA localization: gene expression in the spatial dimension. *Cell* **136**, 719-730, doi:S0092-8674(09)00126-3 [pii] 10.1016/j.cell.2009.01.044 (2009).

2 Besse, F. & Ephrussi, A. Translational control of localized mRNAs: restricting protein synthesis in space and time. *Nat Rev Mol Cell Biol* **9**, 971-980, doi:nrm2548 [pii] 10.1038/nrm2548 (2008).

3 Lawrence, J. B. & Singer, R. H. Intracellular localization of messenger RNAs for cytoskeletal proteins. *Cell* **45**, 407-415, doi:0092-8674(86)90326-0 [pii] (1986).

4 Mingle, L. A. *et al.* Localization of all seven messenger RNAs for the actin-polymerization nucleator Arp2/3 complex in the protrusions of fibroblasts. *J Cell Sci* **118**, 2425-2433, doi:118/11/2425 [pii] 10.1242/jcs.02371 (2005).

5 Blower, M. D., Feric, E., Weis, K. & Heald, R. Genome-wide analysis demonstrates conserved localization of messenger RNAs to mitotic microtubules. *J Cell Biol* **179**, 1365-1373, doi:jcb.200705163 [pii] 10.1083/jcb.200705163 (2007).

6 Eliscovich, C., Peset, I., Vernos, I. & Mendez, R. Spindle-localized CPE-mediated translation controls meiotic chromosome segregation. *Nat Cell Biol* **10**, 858-865, doi:ncb1746 [pii] 10.1038/ncb1746 (2008).

7 Sharp, J. A., Plant, J. J., Ohsumi, T. K., Borowsky, M. & Blower, M. D. Functional analysis of the microtubule-interacting transcriptome. *Mol Biol Cell* **22**, 4312-4323, doi:mbc.E11-07-0629 [pii] 10.1091/mbc.E11-07-0629 (2011).

8 Lecuyer, E. *et al.* Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. *Cell* **131**, 174-187, doi:S0092-8674(07)01022-7 [pii] 10.1016/j.cell.2007.08.003 (2007).

9 Mili, S., Moissoglu, K. & Macara, I. G. Genome-wide screen reveals APC-associated RNAs enriched in cell protrusions. *Nature* **453**, 115-119, doi:nature06888 [pii] 10.1038/nature06888 (2008).

10 Holt, C. E. & Bullock, S. L. Subcellular mRNA localization in animal cells and why it matters. *Science* **326**, 1212-1216, doi:326/5957/1212 [pii] 10.1126/science.1176488 (2009).

11 Desai, A., Murray, A., Mitchison, T. J. & Walczak, C. E. The use of Xenopus egg extracts to study mitotic spindle assembly and function in vitro. *Methods Cell Biol* **61**, 385-412 (1999).

12 Gache, V., Waridel, P., Luche, S., Shevchenko, A. & Popov, A. V. Purification and mass spectrometry identification of microtubule-binding proteins from Xenopus egg extracts. *Methods Mol Med* **137**, 29-43 (2007).
13 Gache, V. *et al.* Xenopus meiotic microtubule-associated interactome. *PLoS One* **5**, e9248, doi:10.1371/journal.pone.0009248 (2010).
14 Cross, M. K. & Powers, M. Obtaining eggs from Xenopus laevis females. *J Vis Exp*, doi:890 [pii]
10.3791/890 (2008).
15 Cross, M. K. & Powers, M. Preparation and fractionation of Xenopus laevis egg extracts. *J Vis Exp*, doi:891 [pii]
10.3791/891 (2008).
16 Cross, M. & Powers, M. In vitro nuclear assembly using fractionated Xenopus egg extracts. *J Vis Exp*, doi:908 [pii]
10.3791/908 (2008).
17 Harland, R. M. & Grainger, R. M. Xenopus research: metamorphosed by genetics and genomics. *Trends Genet* **27**, 507-515, doi:S0168-9525(11)00136-3 [pii]
10.1016/j.tig.2011.08.003 (2011).
18 Hellsten, U. *et al.* The genome of the Western clawed frog Xenopus tropicalis. *Science* **328**, 633-636, doi:328/5978/633 [pii]
10.1126/science.1183670 (2010).
19 Brown, K. S. *et al.* Xenopus tropicalis egg extracts provide insight into scaling of the mitotic spindle. *J Cell Biol* **176**, 765-770, doi:jcb.200610043 [pii]
10.1083/jcb.200610043 (2007).
20 Godfrey, E. W. & Sanders, G. E. Effect of water hardness on oocyte quality and embryo development in the African clawed frog (Xenopus laevis). *Comp Med* **54**, 170-175 (2004).
21 Groisman, I. *et al.* CPEB, maskin, and cyclin B1 mRNA at the mitotic apparatus: implications for local translational control of cell division. *Cell* **103**, 435-447, doi:S0092-8674(00)00135-5 [pii] (2000).
22 Budde, P. P., Desai, A. & Heald, R. Analysis of microtubule polymerization in vitro and during the cell cycle in Xenopus egg extracts. *Methods* **38**, 29-34, doi:S1046-2023(05)00157-X [pii]
10.1016/j.ymeth.2005.07.008 (2006).
23 Kalab, P., Pu, R. T. & Dasso, M. The ran GTPase regulates mitotic spindle assembly. *Curr Biol* **9**, 481-484, doi:S0960-9822(99)80213-9 [pii] (1999).
24 Gaglio, T., Saredi, A. & Compton, D. A. NuMA is required for the organization of microtubules into aster-like mitotic arrays. *J Cell Biol* **131**, 693-708 (1995).
25 Hughes, J. R. *et al.* A microtubule interactome: complexes with roles in cell cycle and mitosis. *PLoS Biol* **6**, e98, doi:07-PLBI-RA-2882 [pii]
10.1371/journal.pbio.0060098 (2008).
26 Suprenant, K. A., Tempero, L. B. & Hammer, L. E. Association of ribosomes with in vitro assembled microtubules. *Cell Motil Cytoskeleton* **14**, 401-415, doi:10.1002/cm.970140310 (1989).

27 Rebagliati, M. R., Weeks, D. L., Harvey, R. P. & Melton, D. A. Identification and cloning of localized maternal RNAs from Xenopus eggs. *Cell* **42**, 769-777, doi:0092-8674(85)90273-9 [pii] (1985).

Figure1
Click here to download high resolution image

Figure 1

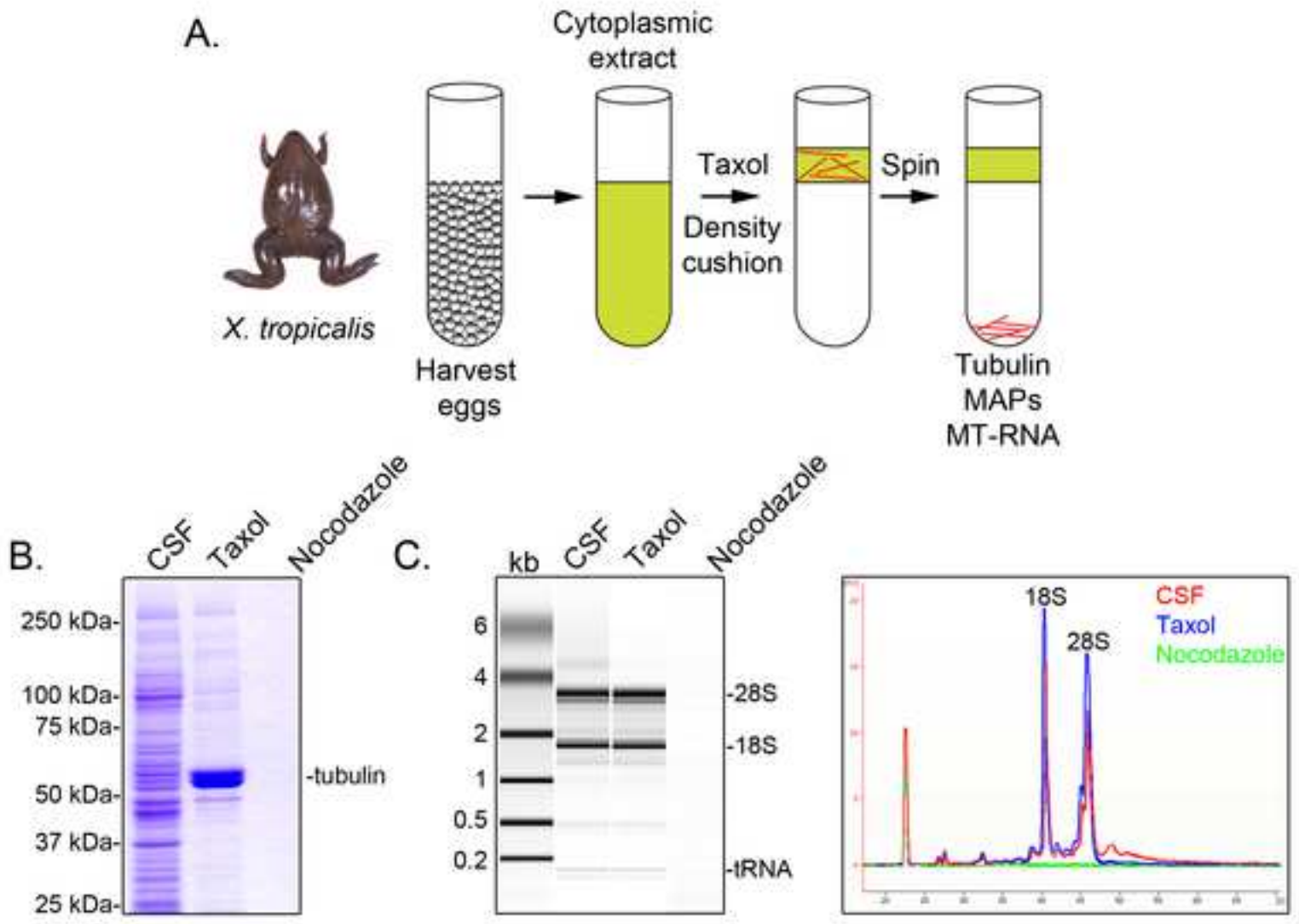

*Table of Reagents/ Materials Used
Click here to download Table of Reagents/ Materials Used: REAGENTS.docx

REAGENTS:

Name	Company	Catalogue Number
Xenopus tropicalis	NASCO	LM00823MX
human Chorionic Gonadotropin	Sigma-Aldrich	CG10
HEPES	Sigma-Aldrich	H4034
EDTA	Sigma-Aldrich	E5134
NaCl	Sigma-Aldrich	S3014
KCl	Sigma-Aldrich	P9541
$MgCl_2$	Sigma-Aldrich	M8266
$CaCl_2$	Sigma-Aldrich	C8106
sucrose	Sigma-Aldrich	S0389
NaOH	Sigma-Aldrich	S5881
EGTA	Sigma-Aldrich	E3889
Leupeptin	Sigma-Aldrich	L9783
Pepstatin	Sigma-Aldrich	P5318
Chymostatin	Sigma-Aldrich	C7268
Cytochalasin D	Sigma-Aldrich	C8273
Gelatin, porcine skin	Sigma-Aldrich	G1890
PIPES	Sigma-Aldrich	P6757
Taxol	Sigma-Aldrich	T7191
Nocodazole	Sigma-Aldrich	M1404
Trizol	Invitrogen	15596-026
L-Cysteine, free base	USB Corporation	14030

EQUIPMENT:

Material Name	**Company**	**Catalogue Number**
1 mL syringes	BD Biosciences	309659
18 gauge needles	BD Biosciences	305195
30 gauge needles	BD Biosciences	305106
Rubbermaid Plastic bucket	Amazon	6306
Beckman Polyallomer 2 x ½ inch Ultracentrifuge tubes	Beckman	326819
15 mL round-bottomed glass centrifuge tubes	Fisher Scientific	45500-15
5 ¾ inch glass Pasteur pipets	Fisher Scientific	13-678-20A
14 mL polypropylene round-bottom tube	BD Biosciences	352059
Sorvall HB-6 rotor	Thermo Scientific	11860
Sorvall RC-6 centrifuge	Thermo Scientific	46910

17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Manuscript #:

Title of Article: Production of Xenopus tropicalis egg extracts to identify microtubule-associated RNAs

Author(s): Judith Sharp + Michael Blower

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☐ Standard Access ☒ Open Access

Item 2 (check one box):

☒ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article

17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT

employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions.

AUTHOR:

Name: Michael Blower

Department: Molecular Biology

Institution: Massachusetts General Hospital

Article Title: Production of X. tropicalis egg extracts to identify microtubule associated RNAs

Signature: 

Date: 10/7/12

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236; or
3) Mail the document to JoVE / Attn: JoVE Editorial / 17 Sellers St / Cambridge, MA 02139

For questions, please email editorial@jove.com or call +1.617.945.9051.

*Rebuttal Comments
Click here to download Rebuttal Comments: 50434_R2_10_18_12_rebuttal.docx

Responses to editorial review:

1. We have now included a written representative results section.
2. We have now included a Figure legend.
3. We have condensed the highlighted portion of the protocol section to occupy ~2 pages. I was not exactly clear about which section needed to be condensed as all of our sections conformed to the word limits outlined in the JoVE template document. If our manuscript is still too long please contact me directly and I will be happy to condense the part that is too long.
4. We have corrected the indicated grammatical mistakes.
5. We have removed usage of second person.

We hope that this submission is now suitable for Peer Review.

*Rebuttal Comments
Click here to download Rebuttal Comments: JoVeResponses.docx

Dear Dr. Henderson,

We appreciate that each of the reviewers found our protocol clear and informative. We have addressed all the points raised be each of the reviewers (see detailed changes below). We hope that our article is now suitable for publication in JoVE.

Please contact me if you need any additional information.

Sincerely,

Mike Blower

Editorial comments:
*Editor modifed the formatting of the manuscript to comply with JoVE instructions for authors, please maintain the current formatting throughout the manuscript. You can find the updated manuscript under "file inventory" and download the microsoft word document.

We have used the editor modified version of the text to make our changes.

*Slight modifications need to be made to the references section of the manuscript, only use "et al" if there are more than 6 authors. There also may be a glitch in the reference manager because some of the reference text appears on a separate line.

We have reformatted the references. We are using the endnote file for JoVE to format the references.

Reviewers' comments:

Reviewer #1:
Summary:
This is a nice methods description and there are no major concerns. Some minor cosmetic revision is recommended.

Minor Concerns:
1.) Introduction, line 11 - are/is

We have corrected the error.

2.) Introduction, paragraph 4 - Xenopus does not benefit. The benefit of using Xenopus...

We have corrected the error. The topic sentence now reads: "For decades *Xenopus laevis* frogs have been a powerful system for the study of developmental and cell biology, owing to the large oocyte size and robust external development[17].

3.) Introduction, paragraph 4 - "the availability of a sequenced genome makes it an attractive model"

We have changed the word “this” to “it” in paragraph 4.

4.) Introduction, paragraph 5 - define CSF first.

The sentence now reads: “In this report we describe a method to make Meiosis II, cytostatic factor-arrested extracts (CSF) from *X. tropicalis*[19].”

5.) Introduction, paragraph 5 - "This provides a powerful method to detect mRNA localization on a genome-wide scale." I have a problem with this statement. The genome-wide scale is OK, but the method only allows to determine, which RNAs are associated with MTs. It does not determine RNA localization in general as the sentence suggests.

We have modified the sentence to read: “This provides a powerful method to detect microtubule-targeted mRNA localization on a genome-wide scale.”

6.) Protocol 1 - I would add "egg harvesting" to the title. Or change it to something like: Generation of Xenopus tropicalis eggs.

We have changed the title to “Generation of X. tropicalis eggs.”

7.) Protocol 1.1 - "injected with hCG at on three successive days" eliminate 'at'

We have made the suggested change.

8.) Protocol 1.4 - What is tankwater? does it contain any salts or is it simply DI water?

We have included our recipe for frog tankwater in the protocol.

9.) Protocol 2.1 - "editing of eggs" What is this?

We have changed “editing” to read: “removal of activated eggs.” In Protocol 2.3, we explain: “In the first XB wash, remove eggs that have escaped CSF arrest by removing lysed, puffy, white, and pseudocleavage eggs.

10.) Protocol 3.2 - be consistent with only rcf or both rcf and rpm throughout manuscript.

We have converted all examples of rpm units to rcf in order to be consistent.

11.) Equipment - adapters are missing.

We have included the part in the equipment list and modified the text replacing "Corex" tubes with glass centrifuge tubes.

12.) Discussion, paragraph 1 - be consistent with abbreviations throughout, hCG vs HCG.

We have made the suggested change.

13.) Discussion, paragraph 2 - "Alternatives could include polymerization using GTP-induced polymerization (a classic technique) or using Ran-GTP as a nmicrotubule polymerizer to mimic the microtubules induced by chromatin-driven spindle assembly." Last line of paragraph should be 'and/or'.

We have made both corrections.

Reviewer #2:
Summary:
In this methods paper Sharp and Blower describe the preparation of Xenopus tropicalis egg extract arrested in meiosis II and the purification of microtubule-associated RNAs. Extracts from Xenopus laevis eggs have been described a long time ago, are well established and have an important place in both historical as well as modern developmental and cell biology. However, Xl does not have a fully sequenced genome while Xt has.
The preparation of Xt egg extract was pioneered by Brown and colleagues in the Heald lab. However, a detailed protocol was never published, hence this manuscript will be of high interest.
The overall structure of the protocol is clear and easy to follow. Therefore, I only have a few, very specific comments and one more general.

Reviewer Concerns:
Specific comments:
(1) Although never having worked with Xt, I remember temperature being crucial not only for the housing and care of the Xt frogs but also for the egg / extract quality. The authors should comment / be more specific about this.

(2) The classic Xl egg extract is spun at 17 000 g, starting the centrifugation at room temperature and cooling down to 4°C. In (2.7.) the eggs are spun at 20°C and not cooled down. Is this because of the nature of the tropicalis eggs or because of the subsequent use of the extract? In (3.2.) any temperature information is missing.

We have modified Protocol sections 1, 2.10, and 3.2 to include more specific temperature information for housing of the frogs and experimental manipulation of

the extract. In addition, we have added text to the first paragraph of the discussion to highlight this point as a major difference between extract preparation methods (see below).

(3) Extracts of Xl eggs are usually sensitive to mechanistic shearing. Do the authors recommend cutting the pipette tips,??

We have not observed mechanistic shearing of the extract when passed through an 18g needle or titurating with a 1 mL pipet tip. We have added text to clarify this point in Protocol section 2.10.

General comment:
This protocol will be particularly interesting for scientists that have so far worked with Xl. Thus, it would be very helpful to the write this protocol in a very analogous manner to the original Xl egg extract protocol. This has been realized for most parts, however, using terms like "packing", ? or particularly pointing out what the main differences / similarities are would improve the manuscript.

The first paragraph of the discussion highlights the main differences between the technical details of preparing egg extracts from *X. tropicalis* versus *X. laevis* frogs. One of the most significant and challenging aspects to working with *X. tropicalis* frogs is to achieve optimal egg laying conditions such that egg extracts still possess biochemical activity. We have added text to elaborate upon this point with regard to egg laying conditions and temperature. In addition, we have added text to clarify what is meant by “packing” of the eggs when the jelly coat is removed.

With regard to format, we have written this manuscript according to the suggested format compliant with JoVE editorial guidelines. In addition, we have taken particular care to provide sufficient detail such that the text, when supplemented with videography, will allow the reader with access to the proper equipment to duplicate the methods precisely.