Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61923R1
Full Title:	Xenopus laevis egg extract preparation and live imaging methods for visualizing dynamic cytoplasmic organization
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Additional Information:	
Question	**Response**
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TITLE:

Xenopus laevis egg extract preparation and live imaging methods for visualizing dynamic cytoplasmic organization

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KEYWORDS:

Xenopus laevis, egg extract, pattern formation, self-organization, live imaging, cell-free systems, cell biology, biochemistry, developmental biology

SUMMARY:

We describe a method for the preparation and live imaging of undiluted cytoplasmic extracts from *Xenopus laevis* eggs.

ABSTRACT:

Traditionally used for bulk biochemical assays, *Xenopus laevis* egg extracts have emerged as a powerful imaging-based tool for studying cytoplasmic phenomena, such as cytokinesis, mitotic spindle formation and assembly of the nucleus. Building upon early methods that imaged fixed extracts sampled at sparse time points, recent approaches image live extracts using time-lapse microscopy, revealing more dynamical features with enhanced temporal resolution. These methods usually require sophisticated surface treatments of the imaging vessel. Here we introduce an alternative method for live imaging of egg extracts that require no chemical surface treatment. It is simple to implement and utilizes mass-produced laboratory consumables for imaging. We describe a system that can be used for both wide-field and confocal microscopy. It is designed for imaging extracts in a 2-dimensional (2D) field, but can be easily extended to imaging in 3D. It is well-suited for studying spatial pattern formation within the cytoplasm. With representative data, we demonstrate the typical dynamic organization of microtubules, nuclei and mitochondria in interphase extracts prepared using this method. These image data can provide quantitative information on cytoplasmic dynamics and spatial organization.

INTRODUCTION:

The cytoplasm constitutes the main volume of a cell and has a distinct organization. The ingredients of the eukaryotic cytoplasm can self-assemble into a wide range of spatial structures,

such as microtubule asters and the Golgi apparatus, which in turn are dynamically arranged and turned over depending on the cell's identity and physiological state. Understanding the spatial organization of the cytoplasm and its link to cellular functions is thus important for understanding how the cell works. *Xenopus laevis* egg extracts have traditionally been used for bulk biochemical assays[1-8], but recent work establishes them as a powerful live imaging system for mechanistic studies of cytoplasmic structures and their cellular functions[9-18]. These undiluted extracts preserve many structures and functions of the cytoplasm, while allowing direct manipulations of cytoplasmic contents not achievable in conventional cell-based models[19,20]. This makes them ideal for characterizing cytoplasmic phenomena and dissecting their mechanistic underpinnings.

Existing methods for imaging extracts require chemical surface modifications, or fabrication of microfluidic devices. One coverslip-based method requires polyethylene glycol (PEG) passivation of glass coverslips[21]. A microemulsion-based method requires vapor deposition of trichloro(1H,1H,2H,2H-perfluorooctyl)silane on glass surfaces[22,23]. Microfluidic-based systems allow precise control of the volume, geometry and composition of extract droplets, but require specialized microfabrication facilities[11,12,24].

Here we introduce an alternative method for imaging egg extracts that is easy to implement and utilizes readily available, low-cost materials. This includes preparation of an imaging chamber with a slide and a coverslip coated with fluorinated ethylene propylene (FEP) tape. The chamber can be used for imaging extracts with a variety of microscopy systems, including stereoscopes and upright and inverted microscopes. This method requires no chemical treatment of surfaces while achieving similar optical clarity obtained with existing glass-based methods discussed above. It is designed to image a layer of extracts with a uniform thickness across a 2D field, and can be easily extended to image a 3D volume of extracts. It is well suited for time-lapse imaging of collective cytoplasmic behavior over a large field of view.

We have used interphase-arrested egg extracts to demonstrate our imaging method. The extract preparation follows the protocol of Deming and Kornbluth[19]. Briefly, eggs naturally arrested in metaphase of meiosis II are crushed by a low speed spin. This spin releases the cytoplasm from the meiotic arrest and allows the extract to proceed into interphase. Normally, cytochalasin B is added prior to the crushing spin to inhibit F-actin formation. However, it can be omitted if F-actin is desired. Cycloheximide is also added prior to the crushing spin to prevent the interphase extract from entering the next mitosis. The extracts are subsequently placed in the aforementioned imaging chambers and placed on a microscope. Finally, images are recorded over time at defined intervals by a camera connected to the microscope, producing time-lapse image series that capture the dynamical behavior of the extract in a 2D field.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Stanford University.

1. Preparation of slides and coverslips

1.1. Apply a layer of fluorinated ethylene propylene (FEP) adhesive tape to a glass slide with a roller applicator. Cut off excessive tape over the edges with a clean razor blade. Prepare FEP tape-coated coverslips in the same way (**Figure 1A**).

1.2. Apply a double-sided sticky imaging spacer to the FEP tape-coated side of the slide. Leave the protective liner on the top unpeeled (**Figure 1A**).

NOTE: The slides and coverslips should be prepared before the experiment. They can be used immediately, or stored in boxes to prevent dust accumulation on surfaces. The well in the imaging spacer is 120 µm deep and has a diameter of 9 mm.

2. Preparation and live imaging of interphase-arrested egg extracts

NOTE: The following protocol is adapted from Deming and Kornbluth[19], Murray[20], and Smythe and Newport[25] with modifications. All steps should be performed at room temperature unless otherwise noted.

2.1. Three to ten days before egg collection, inject mature female *Xenopus laevis* frogs subcutaneously into the dorsal lymph sac with 100 IU of pregnant mare serum gonadotropin (PMSG).

2.2. Sixteen to eighteen hours prior to planned egg collection, inject the frogs from step 2.1 with 500 IU of human chorionic gonadotropin (hCG). Leave frogs at 18 °C in egg laying buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 2.5 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM HEPES, 0.1 mM EDTA, prepare as a 20x stock solution at pH 7.4 and dilute with clean frog tank water to 1x before use) until egg collection.

2.3. On the day of the experiment, collect eggs in a large glass Petri dish and assess egg quality. Discard the eggs that look like white puffy balls or appear in a string (**Figure 1B**). Examine the eggs under a stereoscope, keep the eggs with normal appearance (**Figure 1C**), and discard those with irregular or mottled pigment (**Figure 1D**).

NOTE: This protocol works with eggs collected from a single frog, which typically lays 25 mL of eggs by 16 hours after hCG injection. Usually, a total of 3 to 6 frogs are induced by hCG, and the frog with the highest egg quality is chosen for the extract preparation experiment.

2.4. Transfer eggs to a 400 mL glass beaker, and remove as much egg laying buffer as possible by decanting.

2.5. Incubate the eggs in 100 mL of freshly prepared dejellying solution (2% w/v L-cysteine in water, adjust to pH 8.0 with NaOH) and gently swirl them periodically. After about 3 minutes, pour off the solution, and add 100 mL of fresh dejellying solution.

2.5.1. Continue the incubation until the eggs are tightly packed (no space between eggs), but avoid leaving eggs in the dejellying solution for more than a total of 5 minutes.

2.6. Remove the dejellying solution as much as possible by decanting, and wash the eggs in 0.25x MMR buffer (25 mM NaCl, 0.5 mM KCl, 0.25 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.025 mM EDTA, 1.25 mM HEPES, prepare as a 10x stock solution, adjust to pH 7.8 with NaOH, and dilute in Milli-Q water before use) by adding the buffer, swirling the eggs, and then pouring off the buffer. Repeat a few times until a total of 1 L of the buffer is used for the washes.

2.7. Wash the eggs a few times with a total of 400 mL of egg lysis buffer (250 mM sucrose, 10 mM HEPES, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, make fresh and adjust to pH 7.7 with KOH). Remove eggs with abnormal appearance using a Pasteur pipette between the washes.

NOTE: Eggs with abnormal appearance refer to those that look like white puffy balls (**Figure 1B**), have mottled pigmentation (**Figure 1D**), are deteriorating with a growing white region (**Figure 1E**), or show darkened and contracted pigmented area in the animal hemisphere (**Figure 1F**).

2.8. Using a transfer pipette with its tip cut wide open, transfer the eggs to a 17 mL round-bottom centrifuge tube containing 1 mL of egg lysis buffer. Spin the tube in a clinical centrifuge at 400 x *g* for 15 seconds to pack the eggs.

2.9. Remove as much as possible the egg lysis buffer from the top of packed eggs using a Pasteur pipette.

NOTE: It is important to remove as much buffer from the packed eggs as possible, in order to minimize the dilution of the egg extract. Sometimes, it is necessary to remove some loose eggs along with the residual buffer to accomplish this.

2.10. Determine the approximate volume of the packed eggs, and then add 5 µg/mL aprotinin, 5 µg/mL leupeptin, 5 µg/mL cytochalasin B, and 50 µg/mL cycloheximide directly on top of the packed eggs.

NOTE: Aprotinin and leupeptin are protease inhibitors. Cytochalasin B inhibits actin polymerization, preventing the extract from contracting and gelling[26]. Cycloheximide inhibits protein synthesis, thereby keeping the extract in the interphase of the cell cycle.

2.11. Crush the eggs by centrifuging the tube at 12,000 x *g*, 4 °C, for 15 minutes, in a swinging bucket rotor.

NOTE: At the end of the centrifugation, the eggs should have ruptured and the lysate separated into three main layers: a yellow lipid layer on top, the cytoplasmic extract (also called crude extract) in the middle, and a dark dense layer containing the pigment granules at the bottom (**Figure 1G**).

2.12. Attach an 18-gauge needle to a syringe. With the needle tip bevel facing up, puncture the tube from the side at the bottom of the cytoplasmic layer, and recover the extract by drawing slowly.

NOTE: Draw the cytoplasmic extract slowly to avoid the inclusion of contaminating content from the yellow lipid layer.

2.13. Transfer the recovered cytoplasmic extract to a new microcentrifuge tube and hold it on ice. Use the extract within 1 hour.

2.14. When ready to image, supplement the extract with desired reagents and fluorescence imaging probes.

NOTE: Fluorescence imaging probes label specific cytoplasmic structures so that they can be visualized by a fluorescence microscope.

2.15. Remove the top protective liner from the imaging spacer on the slide prepared in step 1.2, and deposit approximately 7 µL of extract at the center of the well. Immediately apply the FEP tape-coated coverslip with the FEP side facing the extract to seal the well. Quickly proceed to imaging (**Figure 1H,I**).

2.16. Set the slide on an inverted or upright microscope with a motorized stage and a digital camera. Image the extracts at desired spatial positions and time intervals in both bright-field and fluorescence channels.

NOTE: Typically, a 5x objective is used for imaging. The motorized stage enables automated image acquisition at multiple defined spatial positions. Bright-field microscopy visualizes cytoplasmic structures with different degrees of transparency. Fluorescence microscopy visualizes the cytoplasmic structures specifically labeled by the fluorescence imaging probes added in step 2.14. The camera records time-lapse images of these structures, thereby capturing the dynamics of cytoplasmic organization.

REPRESENTATIVE RESULTS:

Xenopus laevis egg extracts can be used to study the self-organization of the cytoplasm during interphase. **Figure 2A** shows results from a successful experiment. We supplemented interphase-arrested extracts with demembranated *Xenopus laevis* sperm nuclei[19] at a concentration of 27 nuclei/µL and 0.38 µM purified GST-GFP-NLS$^{27\text{-}30}$ (fusion protein consisting of glutathione-S-transferase, green fluorescent protein, and a nuclear localization sequence) to allow reconstitution and visualization of interphase nuclei. We also added 1 µM fluorescently labeled tubulin to visualize microtubules, and 500 nM MitoTracker Red CMXRos to visualize mitochondria. Moments after the extract was placed in the imaging chamber, the cytoplasm appeared disorganized. In the course of the next 30 minutes at room temperature, the cytoplasm started to self-organize into cell-like compartments. Microtubule asters grew from centrosomes introduced with the sperm nuclei, and formed microtubule-depleted border zones when met

with microtubules from neighboring asters. GST-GFP-NLS protein translocated into the round interphase nuclei self-assembled from the added demembranated sperm nuclei. Areas depleted of light scattering cytoplasmic components were visible in both bright-field and mitochondria channels (**Figure 2A**, 20 min and 35 min). Mitochondria also became depleted from the borders established by microtubules, and became enriched in isolated compartments that aligned with microtubule compartments. By 60 min at room temperature, a spatial pattern consisting of cell-like compartments should be well-established, with microtubules forming a hollow wreath-like structure, and mitochondria clearly partitioned into each compartment (**Figure 2A**, 53 min).

Figure 2B compares extract performance in imaging chambers with and without FEP tapes on glass. We supplemented interphase-arrested extracts with demembranated *Xenopus laevis* sperm nuclei[19] at a concentration of 27 nuclei/µL and 0.35 µM GST-mCherry-NLS[27-30] (fusion protein consisting of glutathione-S-transferase, mCherry fluorescent protein, and a nuclear localization sequence) to allow reconstitution and visualization of interphase nuclei. We also added 1 µM fluorescently labeled tubulin to visualize microtubules. Differences in dynamics became obvious by approximately 20 min at room temperature. In the chamber made with FEP-taped glass, the extract self-organized into normal cell-like patterns (**Figure 2B**, images in rows 1 and 3). However, in the chamber where glass surfaces were not covered by the FEP tape (unpassivated), the extract showed abnormal bright-field and microtubule patterns that became increasingly disrupted over time (**Figure 2B**, images in rows 2 and 4). No significant differences were observed in nuclear import of the GST-mCherry-NLS protein (**Figure 2B**, images in rows 5 and 6).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematics and photos related to the experimental procedure. (**A**) Schematic diagram for preparing FEP tape-coated glass coverslips and slides. (**B**) *Xenopus laevis* eggs deposited in egg laying buffer, with examples of poor-quality eggs indicated by arrows. Yellow arrows, examples of eggs that look like white puffy balls. Red arrows, examples of eggs that appear in a string. (**C**) Examples of *Xenopus laevis* eggs with normal appearance. (**D**) Examples of poor-quality eggs with irregular or mottled pigment. (**E**) A deteriorating egg with a growing white region. (**F**) An egg that shows darkened and contracted pigmented area, possibly due to parthenogenetic activation. (**G**) The layers formed by ruptured *Xenopus laevis* eggs after the 12,000 x *g* centrifugation in step 2.11. (**H**) Schematics for preparing extract imaging chamber in step 2.15. (**I**) A photo of a prepared imaging chamber with an egg extract inside. (**C**) and (**D**) share the same scale bar at the bottom of (**D**). (**E**) and (**F**) share the same scale bar at the bottom of (**F**). The scale bars in (**B**) (**C**) (**D**) (**E**) (**F**) and (**I**) are approximate.

Figure 2: Interphase-arrested egg extracts self-organize into cell-like compartments. (**A**)Time-lapse montage of self-organized pattern formation in a thin layer (120 µm) of interphase-arrested *Xenopus laevis* egg extract. The extract was supplemented with 27 nuclei/µL of demembranated *Xenopus laevis* sperm nuclei to allow reconstitution of the interphase nuclei. Microtubules were visualized by HiLyte 647-labeled tubulin (shown in magenta), mitochondria by MitoTracker Red CMXRos (shown in red), and nuclei by GST-GFP-NLS (shown in green). (**B**) Self-organized pattern formation in interphase-arrested *Xenopus laevis* egg extracts placed in chambers with and

without FEP-tape covered glass surfaces. The extracts were supplemented with 27 nuclei/µL of demembranated *Xenopus laevis* sperm nuclei to allow reconstitution of the interphase nuclei. Microtubules were visualized by HiLyte 488-labeled tubulin (shown in magenta) and nuclei by GST-mCherry-NLS (shown in green).

Figure 3: Optional secondary seal for the imaging chamber. Schematics for preparing an optional secondary seal with mineral oil to prevent the extract from prolonged contact with air.

DISCUSSION:

Xenopus laevis egg extracts have emerged as a powerful model system for image-based studies of various subcellular structures[10,14-18,21,31-36] and cytoplasmic organization on a whole cell scale[9]. Here we have described a live imaging method suitable for visualizing dynamic cytoplasmic organization in 2D. The effectiveness of the method is demonstrated by representative results.

Several steps are critical to the success of the method. Egg quality is important for extracts, so steps 2.3 and 2.7 are critical. In our experience, the animal hemisphere of high-quality eggs has uniform pigmentation, a distinct white dot at the center (indicative of germinal vesicle breakdown, GVBD), and a clear border with the vegetal hemisphere (**Figure 1C**). The extracts made from high-quality eggs have better performance under our imaging conditions. If more than 5% of the eggs have mottled pigment (**Figure 1D**), have darkened and contracted pigmented area (**Figure 1F**), look like white puffy balls (**Figure 1B**), appear in a string (**Figure 1B**), or show signs of deterioration after the washes in steps 2.6 and 2.7 (**Figure 1E**), then the whole batch of eggs should be discarded. The extracts retain remarkable biological activity because they are essentially undiluted cytoplasm. Therefore, the step that aims to minimize dilution of the extract (step 2.9) is important for the success of the experiment. For imaging, extracts are handled in very small volumes, and will evaporate quickly if in prolonged contact with air. This will negatively affect their activity. Therefore, in step 2.15, after the extract is deposited, it must be sealed with the FEP tape-coated side of the coverslip as soon as possible. Sealing can be visually confirmed by the texture change at the contact site between the adhesive on the spacer and the coverslip. The spacer should be able to create a complete seal if applied properly. However, if an additional seal is desired, mineral oil can be dispensed between the overhang of the coverslip and the glass slide. The oil can form an additional seal around the spacer by capillary action (**Figure 3**). Passivation of glass surfaces can reduce non-specific adsorption of molecules, and it is important for interphase microtubule asters in extracts[21,37]. The application of FEP tape over a glass surface described here appears to offer similar benefits, as suggested by the assembly of normal interphase microtubule asters (**Figure 2**, 6 min). Therefore, step 1.1 is also critical.

We demonstrated the application of an imaging method using interphase-arrested egg extracts following the protocol of Deming and Kornbluth[19]. By default, the protocol supplements the extracts with the actin polymerization inhibitor cytochalasin B to prevent gelation-contraction in the extracts after extended incubation at room temperature[26]. To allow actin polymerization and observe actin dynamics, one can leave out the cytochalasin B in step 2.10 of the protocol[9]. A modification we have made to the Deming and Kornbluth protocol is that we do not supplement extracts with an energy mix to regenerate ATP[19]. This is because in our hands, in extracts

supplemented with this ATP-regenerating mix[19] and sperm nuclei, microtubules occasionally form a crosslinked lattice that interferes with cytoplasmic pattern formation. Therefore, the protocol does not include the step that adds the energy mix to the extracts[19].

The interphase extract protocol relies on crushing eggs in EGTA-free buffer to release them from meiotic arrest (CSF-arrest)[37]. The extracts subsequently progress into interphase, and are kept in interphase by cycloheximide. There are other established methods for preparing interphase extracts. Some methods first prepare extracts that maintain meiotic arrest by crushing eggs in lysis buffer with EGTA[38], and then release the arrest by adding calcium, driving the extract into interphase[21,37,39]. The extracts can be subsequently held in interphase by addition of protein synthesis inhibitors such as cycloheximide[37,39]. Other methods parthenogenetically activate eggs with calcium ionophore (A23187) or electric shock to release them from meiotic arrest, before crushing the eggs in the absence of EGTA[20,28] (reviewed in Field et al.[37]). These extracts can enter interphase but typically will not stay there as they are able to undergo multiple cell cycles. Likewise, well-established methods optimized for preparing extracts with intact actin cytoskeleton have been developed[10,37,39]. The imaging method may be suitable for these types of extracts, but we have not tested it with them.

For the purpose of imaging the internal organization of the cytoplasm, the imaging system presented here are relatively easy to set up, requiring only applying an FEP tape to glass surfaces. It allows assembly of cytoskeletal structures in extracts[9] reported in more sophisticated imaging systems where glass surfaces are passivated with poly-L-lysine-g-polyethylene glycol (PLL-g-PEG) treatment or coated with supported lipid bilayers[10,21]. The method allows the extract layer to form with a defined thickness (determined by the depth of the spacer, which is 120 µm for the system shown in **Figure 1A, 1H, 1I, and Figure 2**). We can adjust the thickness by stacking additional spacers. We have stacked up to 6 such spacers (720 µm thick) and the compartments formed normally. This flexibility enables future applications such as imaging of the extracts in 3D using confocal or light-sheet microscopy.

ACKNOWLEDGMENTS:

We thank J. Kamenz, Y. Chen, and W. Y. C. Huang for comments on the manuscript. This work was supported by grants from the National Institutes of Health (R01 GM110564, P50 GM107615, and R35 GM131792) awarded to James E. Ferrell, Jr.

DISCLOSURES:

The authors have nothing to disclose.

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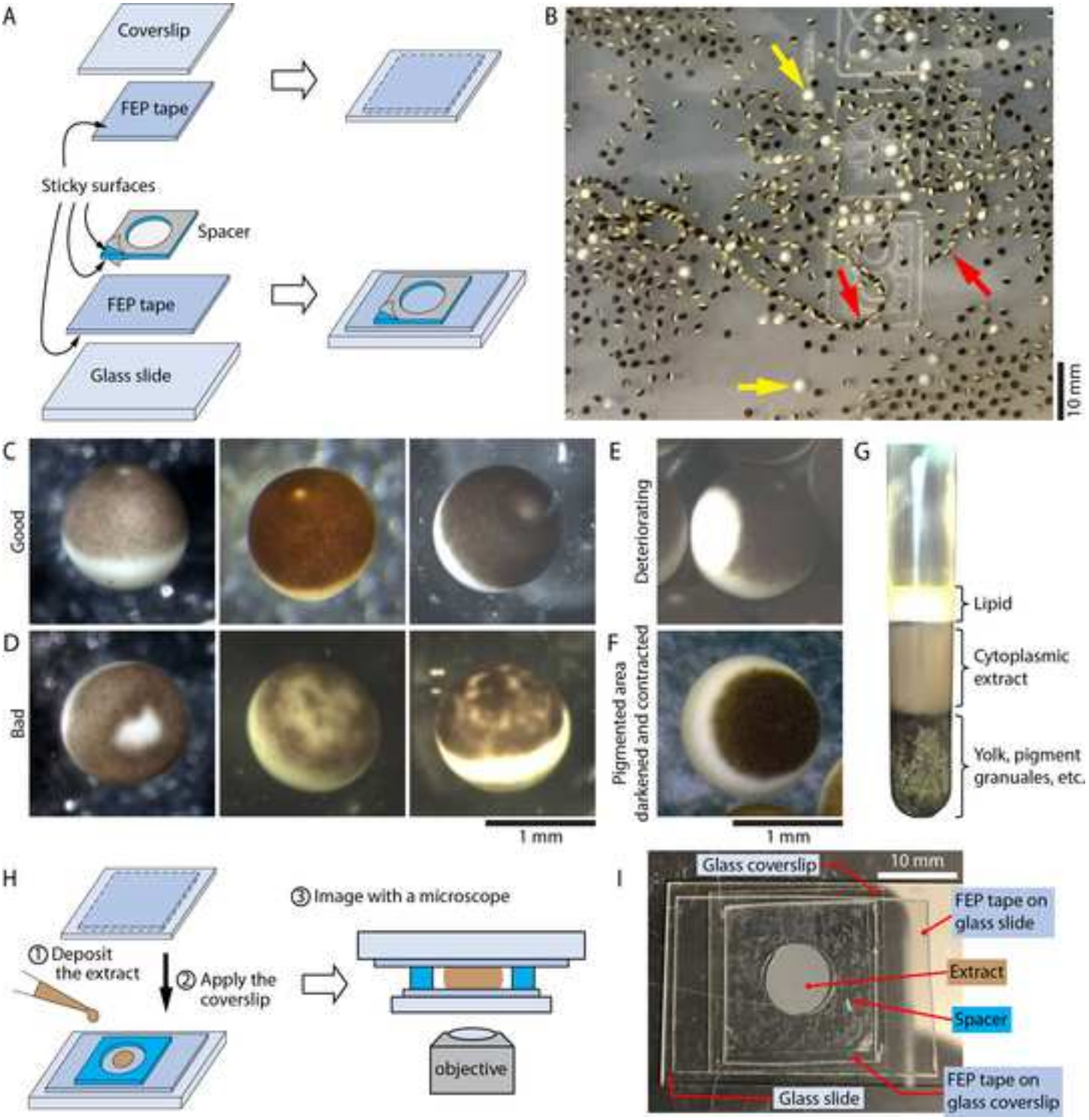

Figure2

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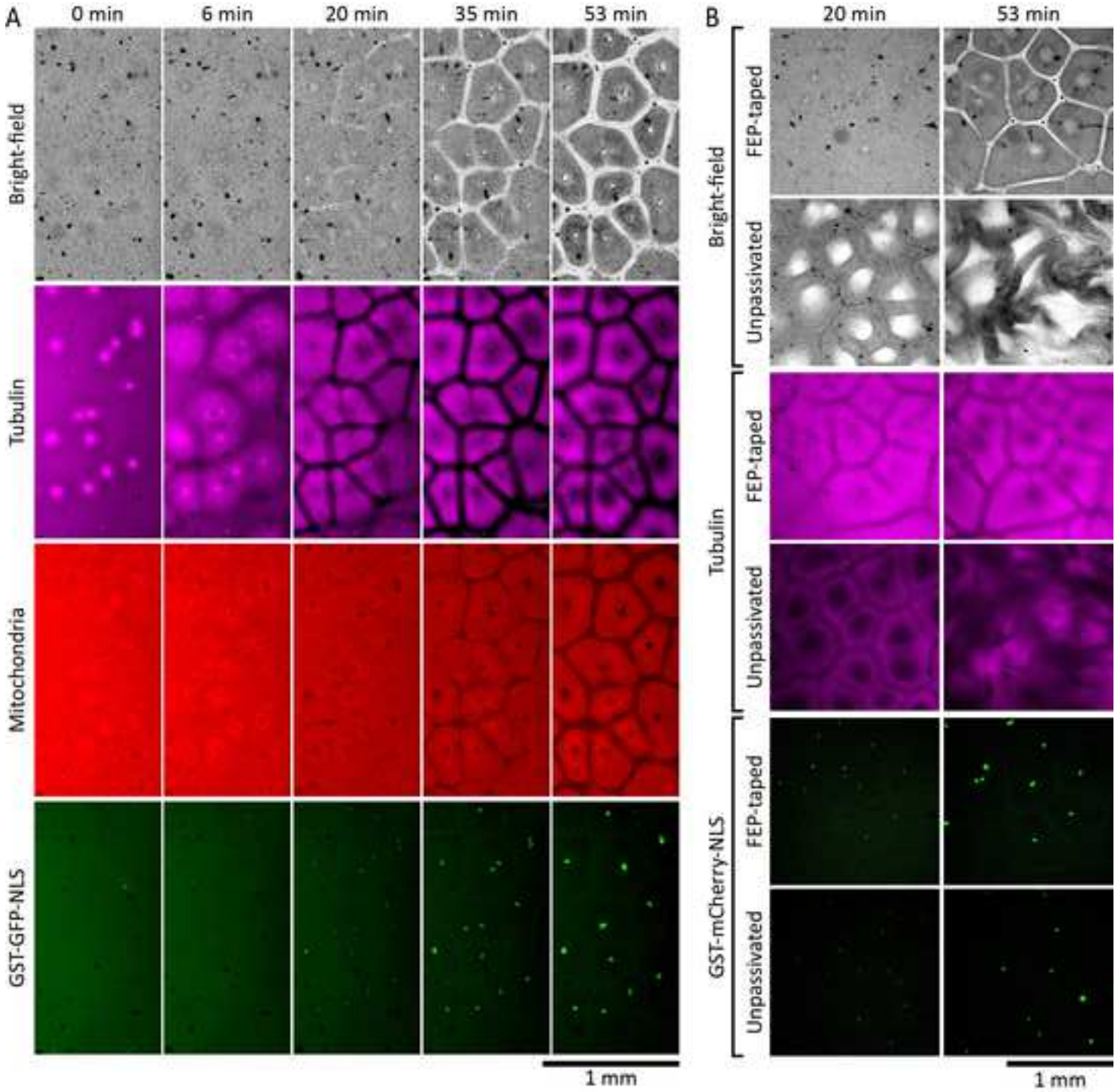

Figure3

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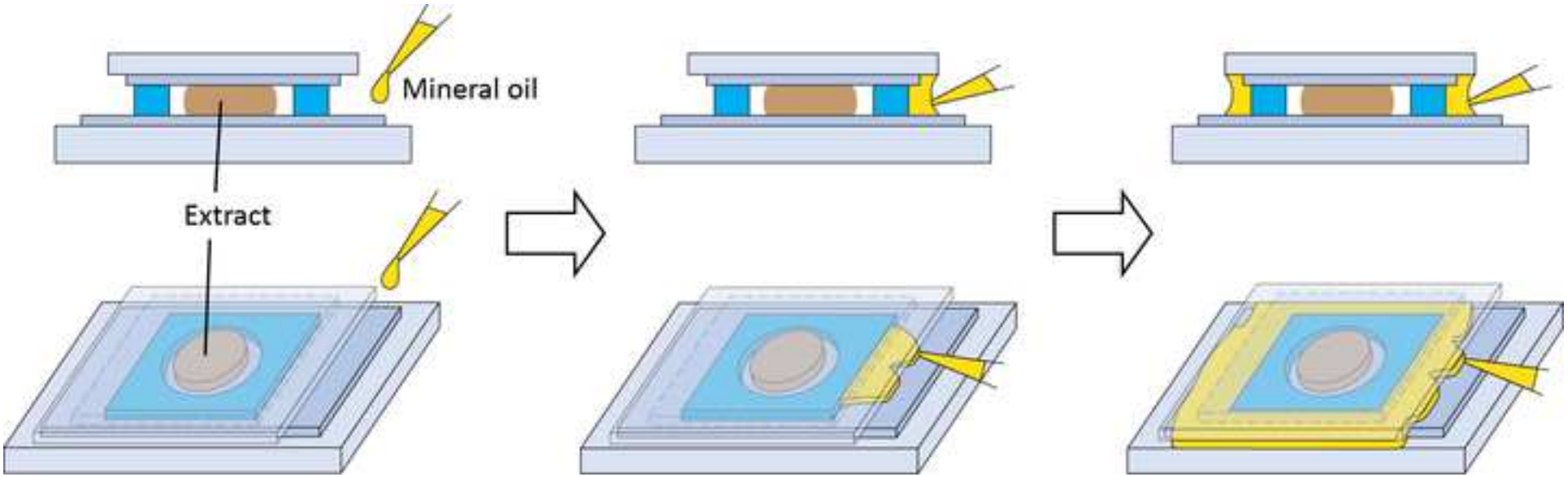

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
17 ml centrifuge tube	Beckman Coulter	337986	
22x22 mm square #1 cover glass	Corning	284522	
Aprotinin	MilliporeSigma	10236624001	Protease inhibitor
Cycloheximide	MilliporeSigma	01810	Protein synthesis inhibitor
Cytochalasin B	MilliporeSigma	C6762	Actin polymerization inhibitor
Female *Xenopus laevis* frogs	Nasco	LM00535MX	
Fluorescent HiLyte 488 labeled tubulin protein	Cytoskeleton, Inc.	TL488M-A	For visualizing the microtubule cytoskeleton
Fluorescent HiLyte 647 labeled tubulin protein	Cytoskeleton, Inc.	TL670M-A	For visualizing the microtubule cytoskeleton
Fluorinated ethylene propylene (FEP) optically clear tape	CS Hyde company	23-FEP-2-5	
Glass Pasteur pipette	Fisher Scientific	13-678-20C	
Human chorionic gonadotropin (hCG)	MilliporeSigma	CG10	
Imaging spacer	Electron Microscopy Sciences	70327-8S	
Leupeptin	MilliporeSigma	11017101001	Protease inhibitor
Microscope slides	Fisher Scientific	12-518-100B	
Mineral oil	MilliporeSigma	330760	
MitoTracker Red CMXRos	Thermo Fisher Scientific	M7512	For visualizing mitochondria
Pregnant mare serum gonadotropin (PMSG)	BioVendor	RP1782725000	
Roller applicator	Amazon	B07HMBJSP8	For applying the FEP tape to the glass slides and coverslips
Single-edged razor blades	Fisher Scientific	12-640	For removing excessive FEP tape
Transfer pipette	Fisher Scientific	13-711-7M	

We thank the reviewers and the editor for their careful review of our manuscript and their helpful comments. The comments and our response are presented below.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have proofread the manuscript and did the suggested checks the best we can.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

We have formatted the manuscript as requested.

3. Please reword the title to exactly reflect the protocol being presented.

We have revised the title as requested. The protocol includes both an egg extract preparation method and an imaging method. The new title reflects both.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

We have made sure that imperative tense is used in the protocol section.

5. Please ensure you answer the “how” question, i.e., how is the step performed?

We have made sure that each step of the protocol clearly describes how the step is performed.

6. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

We have made sure that each step in the protocol contains no more than 3 action sentences.

7. In the protocol please include how do you visualize the dynamic cytoplasmic organization?

We have added ``Notes'' to steps 2.14 and 2.16 to further explain the visualization process.

Briefly, we add fluorescent probes to the extract in step 2.14 to label specific cytoplasmic structures, and then in step 2.16 we use a microscope equipped with a low magnification objective (5x) and digital camera to image these structures, capturing the cytoplasmic organization over time with time-lapse images.

8. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted the relevant steps in the manuscript as requested (approximately 2 pages).

9. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The images in this manuscript have not appeared in previous publications, and therefore no permission is needed.

10. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have updated the Discussion section to cover these aspects where applicable.

11. Figures: Please include scale bars for images taken from a microscope.

We have added additional scale bars as requested.

12. Please sort the materials table in alphabetical order.
We have sorted the materials table in alphabetical order with respect to the names of the reagents. We have also added the fluorescent tubulin protein used in the new figure panel (Figure 2B) to the list.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
A easy method for life cell imaging cellular organization in Xenopus egg extracts. This manuscript is very clearly written, easy to follow. We do not find any point to criticize or where we could suggest an improvement.

We thank the reviewer for reviewing our manuscript and we appreciate the reviewer's positive comments.

Reviewer #2:
Manuscript Summary:
Manuscript presents a protocol based on a published protocol by the authors. It is for a relatively easy preparation of Xenopus egg extract that uses easily available reagents

Major Concerns:
Authors reference multiple papers using various types of Xenopus egg extracts but do not make it clear up front how this extract is different from the ones they have referenced.

We thank the reviewer for pointing out this issue. The introduction has been revised to make it clear how the extract we are preparing is different from CSF and cycling extracts: We are following the protocol of Deming and Kornbluth, and the extract prepared this way is an interphase-arrested extract.

In addition, in the revised Discussion section, we discuss the minor modifications we made to the protocol. For example, the omission of ATP-regenerating mix. We also discuss key differences between our protocol and other extract preparation protocols.

Minor Concerns:
Authors have published a paper with two different interphase extracts used for different purposed, however, they do not distinguish between the two. I think this could be confusing for some people.

The reviewer is correct – we used three types extracts in our previous publication: F-actin inhibited interphase-arrested extracts (following Deming and Kornbluth), F-actin-intact interphase-arrested extracts (following Deming and Kornbluth but with no cytochalasin B added), and cycling extracts (following Murray, 1991, and Chang and Ferrell, 2018). The imaging procedure described in our current manuscript was used for all three types of extracts, and for cycling extracts a different imaging procedure was also used (not the slide/coverslip/FEP tape method described here). In this manuscript, we have chosen to focus on the slide/coverslip/FEP tape method and we describe in detail how we prepare interphase-arrested extracts with F-actin inhibited, but in our experience the slide/coverslip/FEP method works equally well with actin-intact extracts.

Comments for authors: Overall, this reviewer thinks that this protocol will be helpful to the field. I think some clarification is needed between different frog egg extracts and their strengths and weaknesses. I

suggest the authors expand the manuscript to include the ionophore protocol they also used in their paper in reference 9 or distinguish between those two protocols here.

We have added a paragraph in the Discussion section, where key distinctions between these two protocols are discussed.

The calcium ionophore protocol by Murray is used for making cycling extracts. We have some experience using calcium ionophore activation followed by cycloheximide addition to prepare interphase-arrested extracts, but in our hands these interphase-arrested extracts did not perform as well as those prepared following Deming and Kornbluth protocol.

¬ Make it clear what the end product of the protocol is.

We have revised the introduction to convey the message that the end product of the protocol is time-lapse image series that capture the dynamical behavior of the extract (see the last sentence of the Introduction section). This is also conveyed in the new ``Note'' to step 2.16, the last step of the protocol.

The abstract states that this protocol is for making Xenopus egg interphase extract. There is nothing about interphase extracts in the introduction. The introduction is great about referencing papers where Xenopus egg extracts are used--- for both biochemistry and imaging. The papers listed use different kinds of Xenopus egg extracts for different research purposes. This reviewer thinks that it would be helpful to readers to define at the beginning of the paper, either the introduction or just before the protocol starts after the note which references Murray, Kornbluth and Newport.

We have now added a paragraph conveying the message the reviewer suggested at the end of the introduction section.

When discussing Xenopus egg extracts there are several key considerations.
1. Is an extract CSF-arrested (also sometimes called M-phase extract) or has the arrest been released and the extract is progressing to interphase. A minor point is has cycloheximide or another translation inhibitor been added to maintain the interphase state?
2. If the CSF arrest has been released, how was this accomplished? There are 3 common methods; a) the one reported in this protocol, b) addition of calcium to CSF arrested extract and c) calcium ionophore addition to eggs before the crushing spin.
3. Is the actin in the extract intact? ie. was cytochalasin added to the eggs prior to crushing which results in an extract without filamentous actin.

Something like this would be useful. This protocol is for making an interphase extract. The CSF arrest is released during the crushing spin. Cytochalasin is added prior to the crushing spin so there are no actin filaments in the extract and cycloheximide is added so once released, the cell cycle does not proceed

and the extract remains in interphase.

We have added this information at the end of the revised introduction section.

An extract that has cytochalasin added before the crushing spin (actin filament minus) is different from an extract where cytochalasin has not been added (actin intact). Even actin intact extracts where cytochalasin is added after collection can look different from actin filament minus extracts. Some of key information (like the cytochalasin and cycloheximide addition) is in the methods section with a note explaining what they are for which is great but this reviewer thinks it would be very helpful to readers to put this information up front before starting the protocol.

We have made the changes as suggested.

The original Murray protocol for CSF extract contains a buffer with EGTA which chelates calcium this is key to maintain cell cycle the arrest. When a sperm enters an egg, calcium triggers the release of the arrest. This biology is used in all the biochemistry used to keep or release the arrest. The lysis buffer used in this manuscript does not contain EGTA so the release starts immediately upon the start of the crushing spin*, possibly during the washing steps. Most of the papers referenced in this protocol are either CSF arrested extracts or use calcium addition to CSF- extracts to release the arrest -an advantage here is that the cell cycle starts a known time—these usually do not add cycloheximide so the cell cycle clock is running. Another advantage of the calcium addition protocol is that a good CSF extract holds its arrest and remains useable for many hours. Thus, multiple interphase reactions can be made and imaging can be done with the same extract for a longer period of time. A disadvantage or this extract and I think also of the one described in this manuscript is that only one cell cycle can be observed.

These are very good points. We do not have much experience with imaging the interphase extracts prepared from calcium-treated CSF extract, but we expect the imaging method will work for those extracts.

This reviewer is not indicating that one extract is better than another but they are different serve different purposes. This needs to be clear in case readers start comparing results from the various papers referenced here. These authors use two of the three methods for making interphase extracts in their Science paper—this protocol is one of them, with the other being calcium ionophore addition. It is this reviewer's understanding that the calcium ionophore protocol is best if one wants to make cycling extracts and indeed in reference 9, they use the ionophore extract to image multiple cell cycles. This is the most exciting part of the paper. Why do they not include this extract in this protocol? In reference 9 they use the two different extracts for different experiments and use imaging techniques for each. If they found that calcium ionophore is necessary for cycling they should distinguish indicate the difference in this protocol -i.e. this interphase extract is easy to make but it will not perform multiple cell

cycles.

As the reviewer points out, we used a different imaging setup for cycling extracts. Here we chose to focus on the imaging setup that was relied on most heavily in the 2019 paper. We have revised the Introduction and Discussion sections to make it clear that the extract used in the current manuscript is interphase-arrested and will not perform multiple cycles.

Actually, I think they should include the ionophore preparation in this manuscript and discuss the advantages or disadvantages of each. The goal of this type of document is to allow people to duplicate another lab's work. Since they use two different interphase extracts in their paper either report them both here or distinguish between them so people do not get confused.

We have now made it clear in the introduction that we use interphase-arrested extracts prepared following the protocol of Deming and Kornbluth. We also added more discussion in the Discussion section to highlight some key differences between these two types of extracts. The extracts described in this manuscript are released from meiotic arrest during a crushing spin and thus not by calcium ionophore. The extracts are kept in interphase by cycloheximide and thus will not progress through multiple cycles.

¬ Perhaps discuss a few more key differences between the extracts

We have included a new paragraph discussing the differences in the Discussion section as suggested.

Then in the discussion some information putting this extract in perspective to others would be useful. It is fine to mention that the Ferrell extract only used two protease inhibitors, however as mentioned above*, the fact that the lysis buffer does not contain EGTA, which is a key ingredient to most other extract buffers used in the crushing spin in the papers referenced is perhaps more important than the difference between 2 or 3 protease inhibitors which the authors discuss.

We have included a new paragraph in the Discussion section to clarify key differences between our interphase extract protocol and other established protocols, particularly how CSF-arrest is released and interphase is achieved.

In addition, we have removed the discussion of protease inhibitors as reviewer 3 also raised concerns about it.

¬ I like that they make clear some of the important points for making the extract. The pictures of the defective eggs are great. The points mentioning not to dilute the extract and also not to image for longer that 60 min are important.

We thank the reviewer for the positive comments.

¬ I am confused by Figure 1I—where is the extract in that figure? What is the diameter of the holes in the spacer? If the spacer is 120 um thick and 7 ul of extract is placed the center of the well created by the spacer does spread out as indicated in Figure 1H ? In Figure 1I there are two examples of chambers. There is an oblong shaped object inside the well against the right side of the right chamber—is that extract? The left chamber has a similar object at the bottom. Depending upon the diameter of the hole I find it hard to imagine 7 ul of extract spreading out within the well and maintaining a thickness of 120um.

We have now replaced Fig 1I with a more representative photograph, included a scale bar, and annotated relevant objects to make sure the illustration is clear. We added the dimensions of the spacer in the ``Note'' to step 1.2 to improve clarity. We have also updated the materials table so that the glass slide listed there match the one shown in the updated photograph.

To answer the reviewer's questions:

In Fig 1I, the extract is the light brown substance that takes up most of the volume of the imaging chamber. The oblong shaped objects next to the walls of the chambers are air pockets collapsed from the interstitial air surrounding the once centrally localized extract droplet. Sometimes during transportation, the droplet can be displaced off the center, resulting in the scenario seen in Fig 1I. When extracts are deposited in the well, the hydrophobic nature of the FEP coating will cause it to bead up without spreading and flattening quickly. We then directly apply the FEP-taped coverslip to seal the chamber as indicated by Fig 1H, flattening and spreading the droplet in the process. As a result, the final form of the extract droplet is a column of liquid with a defined height (i.e. that of the spacer) sandwiched between two flat surfaces. The diameter of the hole in the spacer is 9 mm and the thickness is 0.12 mm according to the manufacturer. Given these measurements, when approximately 7 µl extract is placed at the center of the well, the droplet will form a near-cylindrical column with a height of 120 µm (i.e. that of one spacer) and a radius of about 4.3 mm. There should be approximately 9/2 – 4.3 = 0.2 mm of clearance between the edge of the extract droplet and the chamber well, if the former is perfectly centered in the chamber. The relevant objects are now annotated and a scale bar is included in the new photograph in Fig 1I.

¬ The spacing between items in the Table of materials is messed up.It is often hard to follow across to the right and the 3 items -----Material, company and catalog number are not aligned. Putting a space between each item should help with that.

We have now left-aligned the text in each column, and included both horizontal and vertical borders in the table so that items are easier to follow.

Reviewer #3:

Manuscript Summary:

This protocol manuscript by Cheng & Ferrell describes the experimental approach to construct chambers

to image the organization of cell-free cytoplasmic extracts derived from the eggs of the African clawed-frog Xenopus laevis using fluorescence microscopy. The work stems from a recent manuscript by the same authors demonstrating that these extracts can self-organize to form a beautiful tessellated array of cell-like compartments within these chambers. The authors construct these chambers by covering a coverslip and glass slide with commercially available FEP tape, serving as the top and bottom of chambers made by sandwiching an adhesive spacer with a hole cut out of it. The use of FEP tape as an alternative to the relatively cumbersome, costly, and time-consuming alternative approaches typically used to passivate glass surfaces will undoubtedly be of tremendous utility to the field, and this is perhaps the most worthwhile aspect of this protocol. However, the construction of these devices is rather straightforward and, in fact, little of this protocol is actually dedicated to describing their assembly. Instead, the bulk of the text is geared toward explaining how to generate cell-free interphase extracts from X. laevis eggs, and the literature is so replete with protocols about the preparation of egg extracts that why this one is warranted now should be better justified. This concern, and several others detailed below, will need to be addressed before this manuscript is suitable for publication.

Major Concerns:

1. The authors should better explain why the field needs another protocol about the preparation of cell-free extracts from X. laevis eggs. I certainly understand that there are nuances in extract preparation and that the extracts described here are specifically non-cycling, interphase extracts, but the manuscript would be significantly improved if some comparison and contrast between recently published protocols about describing X. laevis egg extract preparation from, e.g. the Mitchison & Heald labs, was included.

We have added a new paragraph in the Discussion section to discuss the key differences between our extract protocol and established protocols from the Mitchison lab and the Heald lab in the context of interphase extract preparation.

The reviewer is correct – the egg extract protocol here is not the main contribution of the manuscript. It helps demonstrate the imaging method for visualizing the cytoplasmic self-organization phenomenon.

The main contribution of this manuscript is the imaging method:

(1) A quick, easy and cost-effective way to coat glass surfaces.

(2) An easy way to make a sealed imaging chamber for the extracts. In this system the extracts form a layer with uniform thickness, and this thickness can be adjusted by varying spacer height. This chamber is suitable for many microscope systems.

2. The authors set up what I consider to be a strawman argument about the timeliness of this work, in that they seem to imply that live-cell imaging of extracts is "emerging", i.e. that it is a recent development in the evolution of these extracts from limited utility as a biochemical system. This could simply be a matter of semantics, however, many labs have been using live-cell imaging in X. laevis extracts for the last few decades. Although the authors do provide references, there is a conspicuous

gap in publication dates that could easily be filled with several works published during that time which describe live-cell imaging in egg extracts (e.g. much of the work being done during the 1990s and 2000s in a collaboration between the Salmon and Mitchison labs). The authors should consider either toning down this argument or inclusion of additional references.

We have added additional references from Salmon and Mitchison labs in the Introduction and the Discussion sections as the reviewer suggested. (Murray, Desai, and Salmon, 1996; Desai et al., 1998; Mitchison et al. 2004; Mitchison et al., 2005)

3. The protocol would be improved if the authors make a stronger argument for their form of passivation over existing alternatives. Though the authors do include a brief paragraph describing other passivation methods, it seems that the examples used were chosen in a somewhat arbitrary fashion and the treatment, overall, is far from being comprehensive. I think that this is important because, as I mentioned above, the real utility I see in the work is in the FEP tape-based passivation. I would also suggest that Fig. 2 be modified to facilitate a comparison of extract cellularization in chambers without passivation or with some other widely used, but perhaps less effective form of passivation.

We have carried out new experiments to compare extract dynamics in chambers with and without FEP tape-based passivation. The results clearly show that without FEP tape the cell-like compartments are more poorly organized. These new data have been presented as Figure 2B. We have also updated the main text, the legend to Figure 2, and materials table accordingly.

Minor Concerns:

1. As written, the title of the protocol is misleading as it suggests the protocol will be focused on the imaging of the prepared extracts, consider "Preparation of Xenopus laevis egg extracts to visualize dynamic cytoplasmic organization by live imaging" instead.

The main contribution of this manuscript is the imaging method. However, per the request of the editor inviting this submission we have also included the extract preparation procedure we used in our related publication in 2019, so that together the procedures (imaging method + extract preparation) will yield the desired final product – the time-lapse images.

To address the reviewer's concern, we have changed the title to "*Xenopus laevis* egg extract preparation and live imaging methods for visualizing dynamic cytoplasmic organization".

2. I may have simply missed it, but I could not find the number of frogs that were used in protocol. I am not sure it's critical, but at some threshold number of frogs the volumes of buffers used for some of the steps would have to be adjusted.

We have added a ``Note'' to step 2.3 to clarify the frog usage. We typically induce 3 to 6 frogs for egg production, select the frog with eggs of the highest quality, and use only eggs from that frog for extract preparation.

3. The authors should reference work from Field and Mitchison the first time actin-based gelation and contraction of extracts is mentioned in the text (I should note that this reference is included later in the manuscript).

We have added the reference (Field, Wühr, Anderson, Kueh, Strickland, and Mitchison, 2011) in the ``Note'' to step 2.10, where actin contraction is first mentioned.

4. The schematic in Fig. 1h (part 3) should be changed as it seems to suggest that imaging through the thick glass slide is possible (certainly with a low power, low NA objective). It this was the intent, fine. If the intent was to suggest that the slide could be mounted on either an upright or inverted microscopy, then it should be changed to better convey this message.

We have made the changes to Figure 1H as suggested. In the new illustration the objective is imaging through the thinner coverglass side of the assembly.

5. The fluorescent images in Fig. 2 seem to have some sort of distortion around their edges. This should be corrected before publication.

We have double checked the uploaded original images in Figure 2 but could not identify any distortion. All of the images in the fluorescence channels match the original monochrome images obtained by the camera.

We just noticed that the image quality was greatly reduced and some artifacts appear around the edges in the manuscript PDF preview generated by the online submission system. Perhaps something happened to the images as a result of format conversion in the online submission system, but here everything looks okay.

6. The logic in lines 270-272 is no clear to me. Here the authors state "Finally, since we demonstrate our imaging methods with interphase extracts, the protease inhibitors we use are standard for interphase extracts15,21, and are somewhat different from those used for cycling extracts 16,21,23 and cytostatic factor-arrested extracts16." What is your basis that your protease inhibitor cocktail should be the standard? The implication is that because your experiments worked, your protease cocktail should be the standard, which is not necessarily true. Mitchison's group has used different preparations of interphase extracts, different protease inhibitors, and different passivation methods to generate "normal" asters. Furthermore, to attribute your success to the protease inhibitors used seems to be an overstatement and reach as far as a causality link.

We have removed these statements from the Discussion section.

7. There are a few typos and minor issues with syntax that should be corrected.

We have proofread the manuscript and fixed these mistakes as much as we can.

Reviewer #4:

Manuscript Summary:

The well-written manuscript describes a minimalist method for imaging self-organization of the cytoskeleton and organelles in Xenopus egg cytoplasm. Passivation of glass is a key step for imaging proteinaceous samples and the authors solve this efficiently through use of fluorinated polypropylene tape, eliminating the need for chemical surface treatments. The information is presented clearly and I found it straightforward to follow the steps of protocol. This is a welcome protocol for the community following a 2019 publication from Cheng and Ferrell in Science on topic of self-organization.

Minor Concerns:

I have only a few small notes for the authors.

1. Line 267-269: when describing omission of ATP regenerating system from the extracts the authors mentioned it can negatively impact the performance. Additional clarification should be provided about what aspects of the system are worse. It is quite common in protocols that use Xenopus egg cytoplasm to include this, so more of an explanation would be helpful.

We have updated the Discussion section to clarify this point.

Briefly, when the ATP-regenerating mix described in the Deming and Kornbluth protocol is added to extracts supplemented with sperm nuclei, microtubules will occasionally form a crosslinked lattice that interferes with cytoplasmic pattern formation. We do not know why that happens, but since omitting the energy mix never caused this particular issue, we have chosen to carry out experiments without it.

2. Authors describe a typical time course for self-organization at room temperature. Does this system also perform well at 18 C, but just more slowly.

Yes, the system performs well at 18 C.

Interestingly, self-organized pattern formation proceeds at about the same pace at 18 C as it does at room temperature.

3. Stacking spacers: lines 278-280. I think it would be helpful to indicate the maximum thickness the authors have used in such a setup.

We have added that information in the Discussion section as suggested.

We have used a stack of up to 6 spacers (each 120 μm in height). The cell-like compartments formed normally when examined with a stereoscope.