FINAL SCRIPT: APPROVED FOR FILMING

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# Title: *In vitro* Reconstitution of Cytoskeletal Networks Inside Phase Separated Giant Unilamellar Vesicles (GUVs)

**Authors and Affiliations:**
**Nishu Kanwa*, María Reverte-López*, Petra Schwille**

**Department of Cellular and Molecular Biophysics, Max Planck Institute of Biochemistry**

***These authors contributed equally to this work**

**Corresponding Authors:**
Petra Schwille (schwille@biochem.mpg.de)
**Email Addresses for All Authors:**
Nishu Kanwa (kanwa@biochem.mpg.de)
María Reverte-López (revertelopez@biochem.mpg.de)
Petra Schwille (schwille@biochem.mpg.de)

# Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

**Current Protocol Length**
Number of Steps: 18
Number of Shots: 38

# Introduction

***Videographer: Obtain headshots for all authors available at the filming location.***
**Videographer's Note: Headshots were taken of Maria Reverte-Lopez and Nishu Kanwa. Petra Schwille is on holiday and had not been available.**

**For the statements, the better sound should be on track1 (Lavalier), track2 is unidirectional micro.**

1.1. **María Reverte-López:** Our research explores whether a minimal synthetic cell can replicate key biological functions, focusing on designing membranes that closely mimic the structure and behavior of natural cellular membranes.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What technologies are currently used to advance research in your field?

1.2. **Nishu Kanwa:** Different technologies are employed to make phase-separated vesicles and use these microcarriers for a variety of applications. The technologies are for example double layer cDICE and electroformation.
**Videographer's Note: take 4 is the best in view of the authors**

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What are the current experimental challenges?

1.3. **Nishu Kanwa:** One major challenge is maintaining protein functionality while generating phase separation on the membrane vesicle. Increasing the temperature to reach domain demixing can destabilize proteins and other biomolecules.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:2.12*

***Videographer: Obtain headshots for all authors available at the filming location.***

# Protocol

## 2. Emulsion Transfer Strategy for Cytoskeletal Protein Encapsulation in GUVs

**Demonstrator:** Nishu Kanwa and María Reverte-López

2.1. To begin, obtain vials of biotinylated and non-biotinylated lipid mixes **[1].** Dissolve 50 microliters of each 32 millimolar lipid mix in chloroform **[2].** Then dry them in two glass vials under nitrogen gas flow for approximately 15 minutes **[3]**.

2.1.1. WIDE: Talent holding labelled vials of lipid mix.

2.1.2. Talent pipetting 50 microliters of lipid mix into a glass vial containing chloroform.

2.1.3. Talent placing the vial under a nitrogen gas line and turning on the flow for drying.
Videographer's Note: 2.1.3 A is surplus take, not in the script: turning on the N2-Gas

2.2. Place the glass vials in a desiccator **[1].** Store them under vacuum for approximately 30 minutes to remove any residual chloroform **[2]**.

2.2.1. Talent placing the glass vial into a vacuum desiccator,

2.2.2. Talent vacuum sealing the chamber.

2.3. Now, disperse the dried lipid films in a mixture of 20 microliters of decane and 500 microliters of mineral oil in the same vials to achieve a final lipid concentration of 3.2 millimolar **[1]**. Using a bath sonicator, sonicate the two mixtures at approximately 50 degrees Celsius for 30 minutes **[2]**.

2.3.1. Talent pipetting decane and mineral oil into the glass vial with the dried film. 2.3.2. Talent placing the vial into a bath sonicator set at 50 degrees Celsius.

2.4. Then transfer the lipid-in-oil mixtures into two tubes **[1-TXT].** While the mixtures are incubating, prepare the inner encapsulation mix for proteins **[2]**.

2.4.1. Talent transferring the lipid-in-oil mix into a labeled microtube. **TXT: Incubation: 37 °C, 10 min**
Videographer's Note: 2.4.1A is a surplus take, not in the script (wide and closer) vials into the incubator

2.4.2. Talent setting up workstation and gathering reagents for protein encapsulation mix.

2.5. To prepare the FtsZ *(F-T-S-Z)* protein mixture, add the listed reagents to a final volume of 10 microliters, in a tube **[1-TXT]**. Keep the mixture on ice until use **[2]**.
Videographer's Note: For 2.5.1, 2.6.1 and 2.7.1 it was not clear for me, what to take. So

I shot one scene completely and made some single shots to shorten it, if you wish. I hope this is pleasing you.

2.5.1. Talent pipetting specified volumes of reagents into a microtube. **TXT: FtsZ: Filamenting temperature-sensitive mutant Z**
**AND**
TEXT ON PLAIN BACKGROUND:
FtsZ Protein Mixture:
0.87 µL of 23 µM FtsZ-YFP-mts
1.31 µL of 381 g/L Ficoll70
1.11 µL of 271 g/L BSA
1 µL of 25 mM GTP to 5.71 µL FtsZ reaction buffer (RB)

Final volume of the solution is 10 µL containing 2 µM FtsZ-YFP-mts, 50 g/L Ficoll70, 30 g/L BSA, and 2.5 mM GTP
*Video Editor: Please play both shots side by side*

2.5.2. Shot of the tube being placed on ice.

2.6. For the encapsulation of actin bundles, first prepare 10 microliters of the actin master mix containing 86% G-actin, 10% Atto488 *(Att-oh-Four-Eighty-Eight)*-actin, and 4% biotinylated actin in water **[1].** Keep the mix on ice and protect it from light **[2]**.

2.6.1. Talent pipetting actin proteins and water into a microtube.
**AND**
TEXT ON PLAIN BACKGROUND:
Actin Master Mix (35.42 µM):
6.39 µ L of 2 g/L G-actin
1.48 µL of 1 g/L Atto488-actin
1.19 µL of 0.5 g/L biotinylated actin
0.9 µL H2O
*Video Editor: Please play both shots side by side*

2.6.2. Talent placing the tube on ice and covering it with foil.

2.7. Just before use, prepare the final actin solution by adding, in order, iodixanol, water, Neutravidin, BSA, 1 Ficoll70, 10x actin polymerization buffer, A-Mix, Fascin, and ATP **[1]**. Mix well and use 5 microliters for encapsulation **[2]**.

2.7.1. Talent sequentially pipetting all reagents into a microtube.
**AND**
TEXT ON PLAIN BACKGROUND:
2.92 µL of 60% Iodixanol
10.25 µL H2O
2.5 µL of 0.1 g/L Neutravidin
0.92 µL of 271 g/L BSA
1.31 µL of 381 g/L Ficoll70

2.5 µL of 10x Actin Polymerization buffer
1.69 µL of 35.42 µM A-Mix
1.65 µL of 9 µM Fascin
1.25 µL of 100 mM ATP
*Video Editor: Please play both shots side by side*

2.7.2. Talent mixing the tube gently and retrieving 5 microliters using a pipette.

2.8. To encapsulate FtsZ, pipette 500 microliters of FtsZ reaction buffer into a 1.5-milliliter plastic tube **[1].** Gently add 200 microliters of lipid-in-oil mix to form an oil-water interface **[2]**.

2.8.1. Talent pipetting FtsZ buffer into a 1.5 mL tube.

2.8.2. Talent overlaying lipid-in-oil into a tube without disturbing the interface.

2.9. In a second tube, add 200 microliters of lipid-in-oil mix **[1].** Incubate this tube at 37 degrees Celsius for 10 minutes **[2]**.

2.9.1. Talent pipetting lipid-in-oil into another tube.

2.9.2. Talent placing the emulsion tube in the incubator.

2.10. Next, pipette 5 microliters of the FtsZ protein mix to the incubated lipid-in-oil tube, allowing it to sink as a droplet **[1]**. Gently tap the tube 5 to 6 times until the solution becomes turbid to form an emulsion **[2]**.

2.10.1. Talent adding protein mix to lipid-in-oil. 2

2.10.2. Talent gently tapping the base of the tube.

2.11. Carefully pipette this emulsion on top of the oil-water interface prepared earlier **[1]**. Then centrifuge the sample at 6000 *g* for 30 minutes at 37 degrees Celsius **[2]**. Cool the sample to room temperature for 30 minutes **[3]**.

2.11.1. Talent carefully layering emulsion on top of the preformed oil-water interface.

2.11.2. Talent placing the tube in a preheated centrifuge.

2.11.3. Talent placing the centrifuged tube on a lab bench.

2.12. Now, use a pipette to gently remove the top oil layer, leaving 100 to 200 microliters of solution for imaging **[1]**.

2.12.1. Talent aspirating the top oil layer with a pipette.

2.13. Cut a pipette tip at an angle **[1].** Use it to retrieve 50 to 100 microliters of GUV solution from the bottom of the tube **[2]**.

2.13.1. Talent using scissors to cut a pipette tip.

2.13.2. Talent pipetting the GUVs from the bottom using the cut pipette tip.

2.14. To encapsulate actin directly in a 96-well plate, combine 198 microliters of 2 molar

glucose with 802 microliters of water to prepare an outer glucose solution matching the osmolarity of the inner actin mix **[1]**.

2.14.1. Talent mixing glucose and water in a microtube.

2.15. Passivate a well of a 96-well plate by adding 100 microliters of 10 grams per liter BSA to a well **[1]**. After a 10 to 15-minute incubation, wash the well five times with 100 microliters of outer solution **[2-TXT].** Leave 100 microliters of the final wash **[3]**.

2.15.1. Talent pipetting BSA into the well.

2.15.2. Talent adding outer solution to the wells and pipetting it out. **TXT: Take care not to touch the passivated glass bottom.**

2.15.3. Shot of 100 µL final wash remaining in the well.

2.16. Now gently add 50 microliters of lipid-in-oil mix to the well by resting the pipette on the side wall to ensure correct layering on top of the outer solution **[1]**. In a separate tube, add 100 microliters of lipid-in-oil and incubate **[2-TXT]**.

2.16.1. Talent dispensing the lipid-in-oil from the well edge to form a monolayer.

2.16.2. Talent incubating the lipid-in-oil mixture in a microtube. **TXT: Incubation: 37 °C, 10 min**

2.17. Add 2.5 microliters of final actin solution to the incubated lipid-in-oil tube, allowing it to sink as a droplet **[1]**. Gently tap the bottom of the tube 10 to 20 times until the mixture becomes turbid **[2]**.

2.17.1. Talent pipetting actin mix into lipid-in-oil.

2.17.2. Talent tapping the tube to emulsify.

2.18. Gently pipette the emulsion in the center of the oil monolayer in the well without breaking the interface **[1]**. Then centrifuge the 96-well plate at 200 *g* for 20 minutes at 37 degrees Celsius **[2]**. Allow the plate to cool to room temperature for 30 minutes before imaging **[3]**.

2.18.1. Talent carefully pipetting the emulsion under the oil layer in the well.

2.18.2. Talent placing the 96-well plate into a preheated centrifuge.

# Results

## 3. Results

3.1. Giant unilamellar vesicles or GUVs *(G-U-Vees)* with membranes demixed into liquid-disordered and liquid-ordered domains **[1]**, and a high yield of vesicles sized between 5 and 30 micrometers was obtained **[2]**.

3.1.1. LAB MEDIA: Figure 1B.

3.1.2. LAB MEDIA: Figure 1C. *Video editor: Please sequentially highlight the bars from left to right*

3.2. FtsZ networks were recapitulated within the GUVs and preferentially localized to the liquid-disordered membrane domains in the presence of GTP *(G-T-Pee)* and Ficoll70 *(Fie-koll-Seventy)* **[1]**.

3.2.1. LAB MEDIA: Figure 2. *Video editor: Highlight the left most image and also the rightmost images of both A and B*

3.3. Actin networks, when reconstituted, appeared as thin bundles adhering to the membrane and forming sparse, web-like structures **[1]**. Without biotinylated lipids, actin bundles failed to adhere to the membrane and instead remained rigid and straight within the vesicle lumen **[2]**.

3.3.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the green lines on the leftmost and rightmost images*

3.3.2. LAB MEDIA: Figure 4. *Video editor: Please highlight the leftmost and right most images*

3.4. Under higher centrifugation conditions, vesicles containing actomyosin aggregated into a packed, tissue-like architecture in the production well **[1]**.

3.4.1. LAB MEDIA: Figure 5. *Video editor: Emphasize the purple honeycomb structures in leftmost, 2nd and right most images*

**Pronunciation Guide:**

**1. Cytoskeletal**
**Pronunciation link:**
https://www.merriam-webster.com/dictionary/cytoskeleton
**IPA:** /ˌsaɪ.təˈskɛl.ɪ.təl/
**Phonetic Spelling:** sigh-tuh-skel-uh-tuhl

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**2. Unilamellar**
**Pronunciation link:**
https://www.howtopronounce.com/unilamellar
**IPA:** /ˌjuː.nəˈlæm.ə.lɚ/
**Phonetic Spelling:** yoo-nuh-lam-uh-lur

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**3. Vesicle**
**Pronunciation link:**
https://www.merriam-webster.com/dictionary/vesicle
**IPA:** /ˈvɛs.ɪ.kəl/
**Phonetic Spelling:** veh-sih-kuhl

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**4. FtsZ**
**Pronunciation link:**
https://www.howtopronounce.com/ftsz
**IPA:** /ˈɛfˌtiːˈɛsˈziː/
**Phonetic Spelling:** eff-tee-ess-zee

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**5. Ficoll**
**Pronunciation link:**
https://www.howtopronounce.com/ficoll
**IPA:** /ˈfaɪ.kɔːl/
**Phonetic Spelling:** fie-koll

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**6. GTP**
**Pronunciation link:**
https://www.howtopronounce.com/gtp
**IPA:** /ˌdʒiː.tiːˈpiː/
**Phonetic Spelling:** gee-tee-pee

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**7. Actomyosin**
**Pronunciation link:**
https://www.howtopronounce.com/actomyosin
**IPA:** /ˌæk.toʊˈmaɪ.ə.sɪn/
**Phonetic Spelling:** ak-toh-my-uh-sin

**8. Neutravidin**
**Pronunciation link:**
https://www.howtopronounce.com/neutravidin
**IPA:** /ˌnjuː.trəˈvɪ.dɪn/
**Phonetic Spelling:** nyoo-truh-vi-din

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**9. Biotinylated**
**Pronunciation link:**
https://www.howtopronounce.com/biotinylated
**IPA:** /ˌbaɪ.əˈtɪ.nəˌleɪ.tɪd/
**Phonetic Spelling:** bye-uh-tin-uh-lay-tid

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**10. Iodixanol**
**Pronunciation link:**
https://www.howtopronounce.com/iodixanol
**IPA:** /ˌaɪ.oʊˈdɪk.sə.nɔːl/
**Phonetic Spelling:** eye-oh-dik-suh-nawl

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**11. Atto488**
**Pronunciation link:**
https://www.howtopronounce.com/atto488
**IPA:** /ˈæt.oʊ fɔːrˈeɪ.tiː eɪt/
**Phonetic Spelling:** at-oh four-eighty-eight

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**12. Fascin**
**Pronunciation link:**
https://www.howtopronounce.com/fascin
**IPA:** /ˈfæ.sɪn/
**Phonetic Spelling:** fa-sin