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Title: Mechanical Separation and Protein Solubilization of the Outer and Inner Perivitelline Sublayers from Hen's Eggs

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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? Yes

   If **Yes**, can you record movies/images using your own microscope camera?

   No

   If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit (through a camera port or one of the oculars). Please list the make and model of your microscope.

   Binocular dissecting microscope Vision Engineering, France Model Mantis Elite

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

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

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

Current Protocol Length

Number of Steps: 13
Number of Shots: 30

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Mégane Bregeon:** This protocol provides a step-by-step procedure to sample the perivitelline membrane sublayers from avian eggs for further investigation of their physiological role in bird reproduction.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. Vid NOTE: last one

1.2. **Mégane Bregeon:** The sampling of the perivitelline membrane has been optimized at each step to limit any structural and molecular damages. The protocol was further developed for in-depth proteomics.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. Vid NOTE: last one

Protocol

2. PL, IPL, and OPL samplings

2.1. Begin by sampling the perivitelline layer, or PL, from a freshly laid unfertilized egg **[1]**. Break the egg and use an egg separator to separate the yolk from the white **[2]**. Remove the chalazae *(pronounce 'kuh-ley-zee')* with small scissors **[3]** and roll the yolk over a filter paper to remove adherent albumen that appears as a transparent but visible structure **[4]**.

2.1.1. WIDE: Establishing shot of talent walking to the lab bench with the egg in hand.

2.1.2. Talent breaking the egg and separating the yolk from the white.

2.1.3. Talent removing the chalazae. Vid NOTE: take 1 : ECU, take 2 : CU

2.1.4. Talent rolling the yolk over a filter paper.

2.2. Immerse the yolk in a crystallizer containing 10 millimolar Tris-HCl *(pronounce 'tris-H-C-L')* at pH 8 that has been previously cooled to 4 degrees Celsius **[1]** and remove the PL area over the germinal disc within a 1-centimeter zone using blunt scissors **[2]**. *Videographer: This step is important!*

2.2.1. Talent immersing the yolk in the crystallizer. Vid NOTE: take 2

2.2.2. Talent removing the PL over the germinal disc.

2.3. Rupture the PL with small scissors inside the buffer **[1]**, then hold the two edges of the ruptured PL with forceps and peel it off the yolk **[2]**. *Videographer: This step is important!*

2.3.1. Talent rupturing the PL. Vid and author NOTE: with 2.2.2, the PL was ruptured as we removed the germinal disc (2.2.2)

2.3.2. Talent peeling the PL off the yolk.

2.4. Rinse the PL several times in baths of 10 millimolar Tris-HCl until no trace of yolk is visible **[1]**. Ensure that the PL is clean, white, and floating in the buffer. Process the PL for sublayer separation or proceed directly to biochemical analyses **[2]**. *Videographer: This step is important!*

2.4.1. Talent rinsing the PL.

2.4.2. Clean and white PL floating in the buffer.

2.5. To separate the OPL and IPL **[2]**, spread the entire sample with the OPL facing up in a plastic Petri dish filled with 10 millimolar Tris-HCl and 50 millimolar sodium chloride and maintain it flat with as few wrinkles as possible **[1]**. ~~**[2]**~~.

2.5.1. Talent spreading the sample in the Petri dish.

2.5.2. ~~Talent covering the sample with solution~~. Author NOTE: Put this step before 2.5.1 (if it was shot). Talent pouring solution in Petri dish.

2.6. Determine the location of the remaining chalazae that are only attached to the OPL **[1]**, then cut the entire PL into pieces of about 2 by 3 centimeters with small scissors **[2]**. *Videographer: This step is difficult and important!*

2.6.1. SCOPE: Location where chalazae are attached to the OPL. Vid NOTE: take 2

2.6.2. Talent cutting the PL into pieces. Vid NOTE: slated 2.6.3

2.7. Mechanically separate the two layers with ultra-precise tip forceps under a dissecting microscope **[1]**. Store the resulting IPL and OPL samples individually in microtubes at negative 80 degrees Celsius until further use **[2]**. *Videographer: This step is difficult and important!*

2.7.1. SCOPE: Talent separating the layers. Vid NOTE: slated 2.6.2, from 4/44/44/00

2.7.1B EXTRA shot to compare the 2 different layers

2.7.2. Talent placing one of the layers in a microtube.

3. Sample Treatment for Protein Solubilization and SDS-PAGE Analyses

3.1. To perform primary protein solubilization, freeze-dry the IPL and OPL samples individually **[1]**. Then, cut approximatively 1 milligram from each sample **[2]** and place it inside a clean microtube with a leak proof screw cap **[3]**. Keep the remaining samples in tightly closed tubes for prolonged storage at negative 80 degrees Celsius **[4]**.

3.1.1. Talent using the lyophilizer.

3.1.2. Talent cutting a sample.

3.1.3. Talent putting the sample in the tube. Vid NOTE: Take 1, CU weigh scale

3.1.4. Talent storing sample tubes in a freezer.

3.2. Mix 1 milligram of each lyophilized sublayer with 400 microliters of 50 millimolar Tris at pH 7 and 500 millimolar sodium chloride **[1]**. Use a mixer-mill twice for 5 minutes at 30 Hertz to disintegrate the structures into microparticles and facilitate protein solubilization **[2]**. Collect 400 microliters of the samples into two clean microtubes **[3]**.

3.2.1. Talent mixing the lyophilized sample with solution.

3.2.2. Talent using a mixer-mill. Vid NOTE: take 2

3.2.3. Talent transferring the sample into a clean microtube.

3.3. To prepare the samples for electrophoresis, add 5x SDS-PAGE *(pronounce 'S-D-S-page')* sample buffer to each 400-microliter sample **[1]** and heat it to 100 degrees Celsius for 5 minutes **[2]**.

3.3.1. Talent adding SDS-page buffer to a sample.

3.3.2. Talent heating the sample. Vid NOTE: CU at the end

3.4. Load a maximum of 20 micrograms of proteins into each lane of a 4 to 20% gradient SDS polyacrylamide gel **[1]** and perform electrophoresis at 120 Volts **[2]**.

3.4.1. Talent loading a sample into the gel. Author NOTE: Since the first wells did not look good, please keep the 3 wells on the right

3.4.2. Talent starting the electrophoresis.

3.5. After electrophoresis, remove the gel from the glass plates **[1]** and stain it with Coomassie Brilliant Blue solution for 30 minutes **[1B]**. Then, de-stain with a solution consisting of 50% water, 40% ethanol, and 10% acetic acid until the gel background appears light blue **[2]**.

3.5.1. Talent transferring the gel into a plate

3.5.1B Added shot: add Coomassie Brilliant Blue solution.

3.5.2. Talent transferring gel into the de-staining solution. Vid NOTE: 2nd part

3.6. Transfer the gel into a Petri dish containing deionized water for de-staining and rehydration **[1]**. Proteins should appear as blue bands with a transparent background **[2]**.

3.6.1. Talent transferring the gel into the dish with water.

3.6.2. Blue bands on the gel.

Results

4. Results: SDS-PAGE Analysis of Separated OPL and IPL from Freshly Laid Unfertilized Egg

4.1. The PL was subjected to various buffers to identify conditions allowing minimal protein loss and optimal sublayer separation **[1]**. Protein release at varying Tris concentrations **[1]**, pH **[2]**, and sodium chloride concentrations are shown here **[3]**.

4.1.1. LAB MEDIA: Figure 1.

4.1.2. LAB MEDIA: Figure 3 A.

4.1.3. LAB MEDIA: Figure 3 B.

4.1.4. LAB MEDIA: Figure 3 C.

4.2. The resulting two sublayers were observed under a dissecting microscope **[1]**. IPL is translucent **[2]** while OPL is dense, cloudy, and whitish **[3]**.

4.2.1. LAB MEDIA: Figure 4.

4.2.2. LAB MEDIA: Figure 4. *Video Editor: Emphasize the IPL.*

4.2.3. LAB MEDIA: Figure 4. *Video Editor: Emphasize the OPL.*

4.3. Following the separation, OPL and IPL were lyophilized independently, and their protein content was completely dissolved using a combination of mechanical grinding, an anionic detergent, a reducing agent, and boiling. The samples exhibited distinct electrophoretic profiles on a polyacrylamide gel **[1]**.

4.3.1. LAB MEDIA: Figure 5.

Conclusion

5. Conclusion Interview Statements

5.1. **Mégane Bregeon:** When attempting this protocol, keep in mind that separation of the sublayers is the critical step of the procedure and must be performed using a binocular microscope and in a buffer containing a minimal concentration of salt.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.6.1, 2.7.1.* Vid NOTE: take 2 , one before last

5.2. **Mégane Bregeon:** To further elucidate their respective physiological function, the PL membrane sublayer samples may be analyzed for histological characterization using electronic microscopy and for functional studies using sperm-binding assays and cell migration.

5.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.4.1, 2.4.2.*