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Title: Detailed Preparation of Ni-NTA Lipid Monolayer Affinity Grids for Cryo-EM Structural Analysis of Soluble Polyhistidine-Tagged Protein Complexes

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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? **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**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 31

Number of Shots: 55

Interviews

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

Authors: Answers are slightly edited in accordance with journal guidelines. Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Clara Lenger:** Our research focuses on addressing limitations at the lab bench during sample preparation for protein structural determination via cryo-EM.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Clara Lenger:** This protocol addresses existing gaps by modifying grid surfaces to selectively capture particles, concentrate dilute samples, and tether proteins from the air-water interface.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Clara Lenger:** This method can be used as a platform for structural analysis of soluble his-tagged protein complexes using cryoEM.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Clara Lenger:** The most important challenge in this protocol is the lipid casting and transfer step, which this publication aims to explain thoroughly.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Clara Lenger:** Following this procedure, proteins can be investigated using several cryo-EM methods, including single-particle analysis, tomography, and cryo-TEM imaging.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

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

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

2. Preparation of Lipid Monolayer and Transfer to TEM Grids

Demonstrator: Clara Lenger

2.1. On a well-lit black benchtop [1], arrange the materials required for the lipid casting setup [2]. Orient the flat arm of each pair of anti-capillary tweezers to the carbon side of a TEM (*T-E-M*) grid [3-TXT]. Clamp the grid along the reinforced outer edge [4]. Repeat the procedure for all TEM grids [5].

2.1.1. WIDE: Talent standing at the well-lit black benchtop **Videographer's NOTE:**
Took 2 takes, not sure if take 1 was in focus

2.1.2. TEXT ON A PLAIN BACKGROUND:

LED lamp
Chilled low-salt buffer
Lidded buffer waste bottle
EtOH
EtOH waste bottle
A bottle of castor oil with a dropper
Lipid mixture
Petri dish
2.5 µL syringe
12 pairs of anti-capillary tweezers
12 TEM grids
Low-lint tissues

2.1.3. Talent orienting the flat arm of the anti-capillary tweezers to the carbon side of a TEM grid. **NOTE: 2.1.3 and 2.1.4 were combined**

2.1.4. Talent clamping the TEM grid along its reinforced outer edge. **TXT: TEM: Transmission Electron Microscopy**

2.1.5. Shot showing multiple TEM grids clamped in anti-capillary tweezers.

2.2. Fill the small Petri dish with chilled low-salt buffer [1]. Place 1 large drop of castor oil at the center of the buffer surface [2].

2.2.1. Close-up shot of the small Petri dish being filling with chilled low-salt buffer.
Videographer's NOTE: 2.2.1 and 2.2.2 were combined, extra takes were separated

2.2.2. Talent dispensing a drop of castor oil onto the center of the buffer surface.

- 2.3. Using the 2.5 microliter syringe, draw 1 microliter of lipid mixture [1]. Lower the syringe needle tip to the center of the castor oil [2]. Dispense the lipid mixture as a droplet hanging from the needle bevel just above the surface [3]. Gently touch the droplet to the castor oil [4].
- 2.3.1. Talent drawing 1mL of lipid mixture into the 2.5 microliter syringe. **Videographer's NOTE: Extra take**
- 2.3.2. CU: Shot of lowering the syringe needle tip toward the center of the castor oil. **Videographer's NOTE: 2.3.2, 2.3.3, 2.3.4 combined**
- 2.3.3. Close-up of a lipid droplet hanging from the needle bevel above the surface.
- 2.3.4. Shot of gently touching the lipid droplet to the castor oil.
- 2.4. Using the LED lamp, confirm the formation of a flat lipid circle measuring 2 to 3 centimeters in diameter and surrounded by a ring of castor oil [1]. If this morphology is not observed, discard the buffer from the casting dish [2]. Rinse the dish with reagent ethanol [3], dry it with low-lint tissues [4], and reapply the low-salt buffer, castor oil, and lipid mixture [5].
- 2.4.1. Close-up shot of illuminated casting dish containing a flat lipid circle measuring 2 to 3 centimeters in diameter and surrounded by a ring of castor oil **Videographer's NOTE: 2.4.1, 2.4.2 combined**
- 2.4.2. Shot of discarding the buffer from the casting dish.
- 2.4.3. Shot of rinsing the casting dish with reagent ethanol.
- 2.4.4. Shot of drying the casting dish with low-lint tissues
- 2.4.5. Shot of reapplying low-salt buffer, castor oil, and lipid mixture to the casting dish
- 2.5. Transfer the lipid monolayer onto a TEM grid by lowering the clamped TEM grid to the center of the lipid cast with the carbon side facing downward [1]. Gently touch the entire carbon surface to the lipid monolayer without submerging or releasing the TEM grid [2].
- 2.5.1. Talent lowering the clamped TEM grid toward the center of the lipid cast with the carbon side facing downward. **Videographer's NOTE: 2.5.1, 2.5.2 combined**
- 2.5.2. Close-up of the entire carbon surface touching the lipid monolayer.
- 2.6. Inspect the lipid grid to confirm complete liquid coverage and absence of castor oil [1]. Discard oily grids and repeat the transfer procedure for all remaining TEM grids [2].
- 2.6.1. Close-up of the lipid grid with complete liquid coverage and absence of castor oil. **Videographer's NOTE: took extra takes**
- 2.6.2. Talent discarding the oily TEM grid. **Videographer's NOTE: took extra takes**

3. Preparation of the Humidity Chamber and Lipid Grid Incubation

- 3.1. Set the cryo-plunger humidity chamber to 4 degrees Celsius [1] and the humidity to 100 percent [2].
 - 3.1.1. Show the temperature being set to 4 degrees Celsius in the cryo-cryo-plunger touchscreen. **Videographer's NOTE: 3.1.1, 3.1.2 combined**
 - 3.1.2. Show the humidity being set to 100 percent in the cryo-plunger touchscreen.
- 3.2. Install blot paper onto the blotting pads and equilibrate for at least 1 hour [1].
 - 3.2.1. Talent installing blot paper onto the blotting pads and leaving it undisturbed.
- 3.3. Place at least 20 milliliters of dilution buffer on ice and chill it for at least 20 minutes [1].
 - 3.3.1. Talent placing a container holding 20 milliliters of dilution buffer onto ice and leaving it on ice.
- 3.4. Prepare the area adjacent to the cryo-plunger [1]. Gather the required materials [2] and place them near the cryo-plunger [3].
 - 3.4.1. Shot of the cryo-plunger workstation and surrounding benchtop area
 - 3.4.2. TEXT ON A PLAIN BACKGROUND:
 - Baking dish
 - 3D-printed tweezer holder
 - Self-sealing film strip, 25–35 cm × 10 cm
 - Aluminum foil strip, 35–45 cm × 30.4 cm
 - Ice
 - Paper towels
 - Tap water
 - 3.4.3. Talent placing the materials near the cryo-plunger.
- 3.5. Position the baking dish lengthwise adjacent to the cryo-plunger and fill halfway with ice [1]. Cover with paper towels and lightly wet it with tap water [2].
 - 3.5.1. Talent positioning the baking dish lengthwise adjacent to the cryo-plunger and filling the baking dish halfway with ice.
 - 3.5.2. Talent covering the ice with paper towels and lightly wetting the paper towels with tap water.
- 3.6. Position the strip of self-sealing film lengthwise on the far half of the baking dish directly over the wet paper towels [1].
 - 3.6.1. Talent placing the strip of self-sealing film lengthwise on the far half of the baking dish directly over the wet paper towels

- 3.7. Position the 3D-printed tweezer holder lengthwise in the center of the baking dish, directly on the wet paper towels and self-sealing film [1].
 - 3.7.1. Talent positioning the 3D-printed tweezer holder lengthwise in the center of the baking dish, directly on the wet paper towels and self-sealing film.
- 3.8. Place the clamped lipid grids in the benchtop humidity chamber over the self-sealing film with the lipid side facing upward [1].
 - 3.8.1. Talent placing the clamped lipid grids over the self-sealing film with the lipid side facing upward in the benchtop humidity chamber.
- 3.9. Apply 2 microliters of sample dilution buffer to each lipid surface [1]. Cover the humidity chamber with aluminum foil to minimize evaporation [2].
 - 3.9.1. Talent applying 2 microliters of sample dilution buffer to a lipid grid.
 - 3.9.2. Talent covering the benchtop humidity chamber with aluminum foil.
- 3.10. Maintain hydration of each lipid grid by applying an additional 2 microliters of dilution buffer whenever less than 1 microliter of liquid remains on the grid [1].
 - 3.10.1. Talent applying additional dilution buffer to a lipid grid.
- 3.11. Equilibrate the hydrated lipid grids in the chilled humidity chamber for at least 15 minutes [1].
 - 3.11.1. Shot of hydrated lipid grids in the chilled benchtop humidity chamber.
- 3.12. Apply 3 microliters of polyhistidine-tagged sample to each hydrated lipid grid [1]. Cover the chamber with foil to minimize evaporation and incubate the grids for at least 15 minutes [2].
 - 3.12.1. Talent applying 3 microliters of polyhistidine-tagged sample to a hydrated lipid grid.
 - 3.12.2. Talent covering the benchtop humidity chamber with aluminum foil and leaving it undisturbed.
- 3.13. Maintain hydration of each lipid grid by applying an additional 2 microliters of chilled dilution buffer whenever less than 2 microliters of liquid remain on the grid [1].
 - 3.13.1. Talent applying additional dilution buffer to a lipid grid.

4. Preparation of the Cryo-plunger Pot

- 4.1. Assemble the cryo-plunger pot, including the spindle used for indirect cooling of the brass cup [1].
 - 4.1.1. Talent assembling the cryo-plunger pot and installing the spindle.
- 4.2. After labeling the cryo-grid boxes, place them inside the cryo-plunger pot [1].

- 4.2.1. Talent placing the labeled cryo-grid boxes inside the cryo-plunger pot.
- 4.3. Fill the cryo-plunger pot with liquid nitrogen [1] and cover it to minimize ice contamination [2].
 - 4.3.1. Talent filling the cryo-plunger pot with liquid nitrogen.
 - 4.3.2. Talent covering the cryo-plunger pot.
- 4.4. Maintain the liquid nitrogen level above the cryo-grid boxes by periodically adding additional liquid nitrogen [1].
 - 4.4.1. Talent adding LN2 to maintain the LN2 level above the cryo-grid boxes.
- 4.5. Cool the metal components and cryo-grid boxes to liquid nitrogen temperature for at least 10 minutes [1].
 - 4.5.1. Shot of the metal components and cryo-grid boxes submerged in liquid nitrogen in the cryo-plunger pot.
- 4.6. Fill the brass cup with a 60 to 40 liquid propane-ethane mixture and equilibrate the mixture to liquid nitrogen temperature for at least 5 minutes [1].
 - 4.6.1. Talent filling the brass cup surrounded by liquid nitrogen with the liquid propane-ethane mixture and leaves it in place

5. Plunge-Freezing of Lipid Grids

- 5.1. Verify that the propane-ethane temperature is below minus 185 degrees Celsius [1] and remove the spindle [2].
 - 5.1.1. Shot of the thermometer probe immersed in the propane-ethane mixture, with the display reading below -185 degrees Celsius
 - 5.1.2. Shot of the spindle being removed from the cryo-plunger pot.
- 5.2. Dispense three 50-microliter drops of dilution buffer in a line onto a secured 10 by 10-centimeter piece of self-sealing film [1].
 - 5.2.1. Talent dispensing three 50 microliter drops of dilution buffer in a line onto the self-sealing film.
- 5.3. Wash 1 clamped lipid grid by sequentially touching the lipid surface to each dilution buffer drop [1]. Release the grid on the final dilution buffer drop [2].
 - 5.3.1. Shot of the lipid surface of the clamped grid sequentially touching each dilution buffer drop.
 - 5.3.2. Shot of releasing the lipid grid onto the final dilution buffer drop.
- 5.4. Using the cryo-plunger tweezers, pick up the floating lipid grid [1]. Mount the grid into the cryo-plunger [2].

- 5.4.1. Shot of the floating lipid grid being picked up with the cryo-plunger tweezers.
- 5.4.2. Shot of the lipid grid being mounted into the cryo-plunger.
- 5.5. Absorb the used buffer drops with a lint-free tissue and discard [1]. Apply the cryo-plunger settings to the lipid grid [2]. Plunge-freeze the lipid grid into the propane-ethane mixture [3].
 - 5.5.1. Talent removing the used buffer drops with a lint-free tissue and discarding it.
 - 5.5.2. TEXT ON A PLAIN BACKGROUND:
Cryo-plunger settings:
 - Wait time: 0 s
 - Total blot: 1
 - Blot force: -10
 - Blot time: 4 s
 - Drain time: 0 s
 - 5.5.3. Talent initiating plunge-freezing of the lipid grid into the propane-ethane mixture.
- 5.6. Transfer the cryo-grid from the propane-ethane cup into an available slot in a cryo-grid box and repeat the plunge-freezing procedure for all remaining lipid grids [1].
 - 5.6.1. Talent transferring the cryo-grid from the propane-ethane cup into a slot in a cryo-grid box.

Results

6. Results

- 6.1. After plunge-freezing, the polyhistidine-tagged *Staphylococcus aureus* glutamate synthase or HIS-tagged GS (*hiss-tagged-G-S*) particles, with an average particle count of 274 particles per micrograph, appeared evenly distributed across the lipid monolayer with minimal or no overlap [1].
 - 6.1.1. LAB MEDIA: Figure 6C. *Video editor: Emphasize the particles.*
- 6.2. Lipid grids exhibited higher particle density [1] relative to non-lipid grids prepared at the same protein concentration [2].
 - 6.2.1. LAB MEDIA: Figure 7. *Video editor: Emphasize panels K and O.*
 - 6.2.2. LAB MEDIA: Figure 7. *Video editor: Emphasize panel G.*
- 6.3. Although particle stacking was observed [1], particles on lipid grids exhibited minimal overlap and remained within a similar focal plane [2].
 - 6.3.1. LAB MEDIA: Figure 7K, O. *Video editor: Emphasize the stacked chains of particles.*
 - 6.3.2. LAB MEDIA: Figure 7. *Video editor: Emphasize panels K and O.*
- 6.4. In contrast, particles on non-lipid grids appeared at substantially different focal depths [1], with aggregation observed at 1 milligram per milliliter [2].
 - 6.4.1. LAB MEDIA: Figure 7C, G. *Video editor: Highlight the black and white arrows and the particles indicated by the arrows.*
 - 6.4.2. LAB MEDIA: Figure 7C. *Video editor: Emphasize clumps of particles.*
- 6.5. Lipid cryo-grids containing 0.05% *N*-dodecyl-beta-D-maltoside or DDM (*D-D-M*) exhibited gaps in the lipid monolayer [1], lipid micelles on the grid surface [2], and reduced particle density [3] relative to the control condition [4].
 - 6.5.1. LAB MEDIA: Figure 8I. *Video editor: Highlight the arrow and the small light patch indicated by the arrow.*
 - 6.5.2. LAB MEDIA: Figure 8K. *Video editor: Highlight the arrow and the big dark grey circled region that the arrow indicates.*
 - 6.5.3. LAB MEDIA: Figure 8. *Video editor: Emphasize panel F.*
 - 6.5.4. LAB MEDIA: Figure 8. *Video editor: Emphasize panel C*
- 6.6. Ice quality varied across grid squares, with regions showing thin or absent ice [1], uneven ice distribution [2], or excessively thick ice [3].

- 6.6.1. LAB MEDIA: Figure 9A. *Video editor: Highlight the dashed circular region in the center of the grid square.*
- 6.6.2. LAB MEDIA: Figure 9B. *Video editor: Highlight the dashed oval region.*
- 6.6.3. LAB MEDIA: Figure 9C. *Video editor: Highlight the white arrow and the large dark region indicated by the arrow.*
- 6.7. Failure to transfer the lipid monolayer resulted in cryo-grids containing empty holes [1] or holes with vitreous ice lacking lipid film and exhibiting few or no particles [2].
 - 6.7.1. LAB MEDIA: Figure 10A. *Video editor: Emphasize the large empty circular hole in the center of the image.*
 - 6.7.2. LAB MEDIA: Figure 10B, C. *Video editor: In 10B, emphasize the large circular hole in the center and the half in the left. In 10C, emphasize the edge line and its light gray center, avoiding the round black structures.*

- 1. Ni-NTA
Pronunciation link: <https://www.merriam-webster.com/dictionary/Ni>
IPA: /'nɪkəl/ (Ni = nickel); /,en ti: 'eɪ/ (NTA)
Phonetic Spelling: nɪk·uhl; en·tee·ay
- 2. Monolayer
Pronunciation link: <https://www.merriam-webster.com/dictionary/monolayer>
IPA: /'mɑː.nouˌleɪ.ər/
Phonetic Spelling: mon·oh·lay·ur
- 3. Cryo-EM
Pronunciation link: No confirmed link found
IPA: /,kraɪ.oʊˌiː'ɛm/
Phonetic Spelling: kry·oh·ee·em
- 4. Polyhistidine
Pronunciation link: <https://www.merriam-webster.com/medical/polyhistidine>
IPA: /,pɑː.liˌhɪs.tɪ'diːn/
Phonetic Spelling: pol·ee·his·tuh·deen
- 5. Biochemistry
Pronunciation link: <https://www.merriam-webster.com/dictionary/biochemistry>
IPA: /,baɪ.oʊ'kem.ə.stri/
Phonetic Spelling: by·oh·kem·uh·stree
- 6. Biophysics
Pronunciation link: <https://www.merriam-webster.com/dictionary/biophysics>
IPA: /,baɪ.oʊ'fɪz.ɪks/
Phonetic Spelling: by·oh·fiz·iks

7. Stereomicroscope
Pronunciation link: <https://www.merriam-webster.com/medical/stereomicroscope>
IPA: /,ster.i.oo'maɪ.krə,skoʊp/
Phonetic Spelling: ster·ee·oh·my·kruh·skohp
8. Microinjection
Pronunciation link: <https://www.merriam-webster.com/medical/microinjection>
IPA: /,maɪ.kroʊ.ɪn'dʒek.ʃən/
Phonetic Spelling: my·kroh·in·jek·shuhn
9. Cryo-TEM
Pronunciation link: No confirmed link found
IPA: /,kraɪ.oo'ti:i:'em/
Phonetic Spelling: kry·oh·tee·ee·em
10. Anti-capillary
Pronunciation link: <https://www.merriam-webster.com/dictionary/capillary>
IPA: /,æn.taɪ'kæp.ə,ler.i/
Phonetic Spelling: an·tye·kap·uh·lair·ee
11. Transmission
Pronunciation link: <https://www.merriam-webster.com/dictionary/transmission>
IPA: /trænz'mɪʃ.ən/
Phonetic Spelling: tranz·mish·uhn
12. Microliter
Pronunciation link: <https://www.merriam-webster.com/medical/microliter>
IPA: /'maɪ.kroʊ,li:tər/
Phonetic Spelling: my·kroh·lee·tur
13. Bevel
Pronunciation link: <https://www.merriam-webster.com/dictionary/bevel>
IPA: /'bev.əl/
Phonetic Spelling: bev·uhl
14. Morphology
Pronunciation link: <https://www.merriam-webster.com/dictionary/morphology>
IPA: /mɔ:r'fɑ:lə.dʒi/
Phonetic Spelling: mor·fol·uh·jee
15. Equilibrate
Pronunciation link: <https://www.merriam-webster.com/dictionary/equilibrate>
IPA: /,i:kwə'laɪ.breɪt/
Phonetic Spelling: ee·kwuh·lye·brayt
16. Incubate
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>
IPA: /'ɪŋ.kjə,beɪt/
Phonetic Spelling: ing·kyuh·bayt
17. Propane-ethane
Pronunciation link: No confirmed link found
IPA: /'proʊ.peɪn'i:θeɪn/
Phonetic Spelling: proh·payn ee·thayn

18. Staphylococcus
Pronunciation link: <https://www.merriam-webster.com/medical/Staphylococcus>
IPA: /ˌstæf.ə.ləˈkɑː.kəs/
Phonetic Spelling: staf·uh·luh·kok·uhs
19. Aureus
Pronunciation link: <https://www.merriam-webster.com/medical/aureus>
IPA: /ˈɔːr.i.əs/
Phonetic Spelling: or·ee·uhs
20. Glutamate
Pronunciation link: <https://www.merriam-webster.com/dictionary/glutamate>
IPA: /ˈgluː.təˌmeɪt/
Phonetic Spelling: gloo·tuh·mayt
21. Synthase
Pronunciation link: <https://www.merriam-webster.com/medical/synthase>
IPA: /ˈsɪn.θeɪs/
Phonetic Spelling: sin·thays
22. Micrograph
Pronunciation link: <https://www.merriam-webster.com/dictionary/micrograph>
IPA: /ˈmaɪ.krəˌɡræf/
Phonetic Spelling: my·kruh·graf
23. Monolayer
Pronunciation link: <https://www.merriam-webster.com/dictionary/monolayer>
IPA: /ˈmɑː.noʊˌleɪ.ər/
Phonetic Spelling: mon·oh·lay·ur
24. N-dodecyl-beta-D-maltoside
Pronunciation link: No confirmed link found
IPA: /ɛn doʊˈdes.ɪl ˈbeɪ.tə diː ˈmæl.toʊ.said/
Phonetic Spelling: en·doh·des·il bay·tuh dee mal·toh·syde
25. Micelles
Pronunciation link: <https://www.merriam-webster.com/dictionary/micelle>
IPA: /maɪˈselz/
Phonetic Spelling: my·selz
26. Vitreous
Pronunciation link: <https://www.merriam-webster.com/dictionary/vitreous>
IPA: /ˈvɪt.ri.əs/
Phonetic Spelling: vit·ree·uhs