FINAL SCRIPT: APPROVED FOR FILMING

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Title: Expansion Microscopy: High-Resolution Fluorescent Imaging with a Conventional Microscope

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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**

Current Protocol Length
Number of Steps: 23
Number of Shots: 53

Introduction

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

1.1. **Eloïse Bertiaux:** We are cell biologists investigating the role of microtubules and associated structures in different model systems. We aim at uncovering their functions in cell organization, division, ciliogenesis and morphogenesis.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.2.1*

What significant findings have you established in your field?

1.2. **Marine Laporte:** Over the past five years, we have contributed to improving the understanding of centriole molecular composition through the use of expansion microscopy, revealing key features of their assembly and function.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2.1*

What research gap are you addressing with your protocol?

1.3. **Eloise Bertiaux:** By combining expansion microscopy with cryo-fixation, our protocol enables high-resolution imaging while preserving near-native cellular ultrastructure, overcoming key limitations of classical super-resolution microscopy.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 6.3.1*

What advantage does your protocol offer compared to other techniques?

1.4. **Marine Laporte:** Expansion microscopy is a low-cost and easy-to-implement method that facilitates access to super-resolution microscopy in all research laboratories.

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

Protocol

2. Cryo-Fixation of Coverslips for Electron Microscopy Using a Cryo-Plunger System

Demonstrator: Marine Laporte

2.1. To begin, use a syringe and a needle to fill each 5-milliliter tube with 1 milliliter of extra-dry acetone under a chemical hood **[1]**. Place the tubes upright in metal rack and put them in liquid nitrogen for freezing **[2]**.

2.1.1. WIDE: Talent using a syringe to fill 5-milliliter tubes with extra-dry acetone under a chemical hood.

2.1.2. Talent placing the acetone-filled tubes upright into a rack and submerging it in liquid nitrogen.

2.2. Fill half of a 30 by 30 centimeters, thick-walled polystyrene box with dry ice **[1]** and then fill the Vitrobot-type dewar with liquid nitrogen until evaporation stops **[2]**.

2.2.1. Talent filling a large polystyrene box with dry ice to the halfway mark.

2.2.2. Talent pouring liquid nitrogen into a Vitrobot-type dewar and checking for cessation of evaporation. **TXT: After 10 to 15 min, refill the dewar with liquid nitrogen if necessary**

2.3. Now, remove the spider from the plunging apparatus **[1]** and fill the metallic plunging chamber with liquid ethane **[2]**. Wait for 10 minutes to allow the ethane to reach equilibrium **[3]**.

2.3.1. Talent removing the spider from the plunging setup.

2.3.2. Talent carefully filling the metal chamber with liquid ethane.

2.3.3. Close-up of the metal chamber stabilizing undisturbed.

2.4. Then, grab a 12-millimeter coverslip with thin tweezers **[1]** and dab it on a tissue paper to soak up any excess medium from the coverslip **[2]**. Hold the coverslip halfway using tweezers fitted with a clamping ring compatible with the cryo-plunger **[3]**.

2.4.1. Talent picking up a 12-millimeter coverslip using thin tweezers.

2.4.2. Talent dabbing the coverslip with tissue paper to absorb excess medium.

2.4.3. Close-up of talent positioning the coverslip in the tweezers with a clamping ring.

2.5. Place the tweezers holding the coverslip into the cryo-plunger holder **[1]** and use Whatman paper to blot away any remaining medium **[2]**. Activate the cryo-plunger to plunge the coverslip into the ethane solution contained in the metal chamber **[3]**.

2.5.1. Talent securing the tweezers into the holder of the cryo-plunger.

2.5.2. Talent using Whatman paper to remove the remaining liquid from the coverslip.

2.5.3. Talent pressing the activation mechanism on the cryo-plunger to plunge the coverslip.
AUTHOR'S NOTE: Merge shots 2.5.2 and 2.5.3 – The unique take called 2.5.2 was done

2.6. Then, quickly transfer the coverslip into the tube containing frozen acetone **[1]**.

2.6.1. Talent immediately placing the freshly plunged coverslip into a tube pre-filled with frozen acetone.

3. Freeze Substitution and Rehydration

Demonstrator: Eloïse Bertiaux

3.1. Incubate the tubes containing the coverslips in dry ice at an angle of approximately 45 degrees **[1]**. Place the closed container on an orbital shaker set to 4 degrees Celsius and agitate overnight to let the temperature rise gradually rise **[2]**.

3.1.1. Talent arranging the tubes at a 45-degree tilt in a dry ice bed.

3.1.2. Talent placing the closed container on an orbital shaker.

3.2. Next, remove most of the dry ice from the box **[1]**. Continue agitation on the orbital shaker for an additional 45 minutes to let the temperature rise from minus 80 degrees Celsius to minus 20 degrees Celsius **[2]**. Briefly open and close each tube to release internal pressure **[3]**.
NOTE: 3.2.2 was moved after 3.2.3 at author's request. Shot numbers have been edited accordingly

3.2.1. Talent scooping out dry ice from the container.

AUTHOR'S NOTE: Shots 3.2.1 and 3.2.3 were merged under 1 unique take called "3.2.1

3.2.2. Talent quickly opening and closing the cap of a tube to release pressure. AUTHOR'S NOTE: Move 3.2.2 after 3.2.3

3.2.3. Talent placing the tubes back on the shaker and starting the agitation.

3.3. Then, transfer each coverslip into a 12-well plate or a suitable container pre-filled with pre-chilled 100 percent ethanol solution **[1]**. After a 5-minute incubation, rehydrate the coverslips through a graded ethanol series **[2-TXT]**.

3.3.1. Talent using tweezers to place frozen coverslips into a 12-well plate with chilled ethanol.

3.3.2. Talent transferring coverslip to the next well. **TXT: EtOH 100% (5 min); EtOH 95% (3 min 2x); EtOH 70% (3 min); EtOH 50% (3 min); EtOH 25% (3 min); H_2O**

3.4. After transferring the coverslips into PBS, place them under a microscope **[1]**. Use fine tweezers to gently scratch the surface and orient all coverslips with the correct side facing up **[2]**.

3.4.1. Talent placing coverslips in PBS.

3.4.2. Close-up of scratching the surface of a coverslip with fine tweezers to adjust its orientation.

4. Protein Anchoring, Gelation and Denaturation

Demonstrators: Léo Krüttli, Marine Laporte & Eloïse Bertiaux

4.1. Place the coverslips into a 4-well plate filled with 0.5 to 1 milliliter of acrylamide and formaldehyde solution **[1]**. Then, incubate the coverslips in a solution containing 1.4 percent formaldehyde and 2 percent acrylamide in 1x PBS for without agitation **[2-TXT]**.

4.1.1. Talent transferring coverslips into wells containing the AA/FA mixture.

4.1.2. Talent placing the plate in a 37-degree Celsius incubator and closing the door. **TXT: 3 - 5 h; 37 °C**

4.2. Next, thaw 10% APS and the gelation solution on ice for 10 minutes before gelation **[1]**.

Prepare a humid chamber using a thin layer of wet tissue and parafilm **[2]**, then store it at 4 degrees Celsius **[3]**. After 10 minutes, place the humid chamber on a cold block for use during gelation **[4]**.

4.2.1. Talent placing labeled tubes of APS, and gelation solution on ice to thaw.

4.2.2. Talent lining a chamber with wet tissue and parafilm.

4.2.3. Talent keeping the chamber at 4 degrees Celsius.

4.2.4. Talent removing and placing the prepared humid chamber on a cold block.

4.3. Now, remove the coverslips from the protein anchoring solution **[1]** and blot away excess liquid using tissue paper in two successive passes **[2]**. Add TEMED and APS to the gelation solution to reach a final concentration of 0.5 percent **[3]** and vortex for 2 to 3 seconds **[4]**.

4.3.1. Talent picking up coverslip from anchoring solution.

4.3.2. Talent placing the coverslip on folded tissue paper.
AUTHOR'S NOTE: 4.3.1 and 4.3.2 shots were merged under 1 unique take called "4.3.1"

4.3.3. Talent adding APS to the gelation solution.

4.3.4. Talent vortexing the gelation mixture briefly.

4.4. Then, pipette two 35-microliter drops onto the parafilm in the humid chamber **[1]** and gently place each coverslip over a drop with the cells facing downward into the gelation solution **[2]**.

4.4.1. Talent pipetting droplets onto the parafilm surface.

4.4.2. Close-up of talent inverting and placing coverslips over the gelation drops.
AUTHOR'S NOTE: 4.4.1 and 4.4.2 shots were merged under 1 unique take called "4.4.1"

4.5. ~~Incubate the setup on ice for 5 minutes to facilitate gel penetration **[1]**. Then~~ Transfer the humid chamber to a 37 degrees Celsius incubator for 30 to 60 minutes **[2]**.

~~4.5.1. Talent placing the chamber on ice.~~
NOTE:Shot deleted by authors

4.5.2. Talent transferring the chamber to an incubator.

4.6. For denaturation, use a biopsy punch tool with a 0.4-centimeter diameter to extract gel pieces **[1]** and place them into a 6-well plate filled with 1 milliliter of denaturation buffer **[2]**.

4.6.1. Talent using a biopsy punch and taking out gel discs.

4.6.2. Talent placing the denaturation buffer containing 6-well plate.

4.7. Agitate the plate for 10 to 15 minutes until the gels detach from the coverslips **[1]** and transfer the detached gel pieces into 1.5-milliliter microcentrifuge tubes filled with fresh denaturation buffer **[2]**.

4.7.1. Talent placing the 6-well plate in a shaker.

4.7.2. Talent transferring detached gels into labeled microcentrifuge tubes.

4.8. Incubate the gels for 90 minutes at 95 degrees Celsius **[1]**. After incubation, transfer the gels into double-distilled water for 10 minutes for washing **[2-TXT].** Measure the diameter of the gels using millimeter paper to evaluate the gel expansion factor **[3]**.

4.8.1. Talent placing microcentrifuge tubes in a heating block or water bath at 95 degrees Celsius.

4.8.2. Talent washing the gels in double-distilled water across three cycles. **TXT: Wash the gel in ddH$_2$O 3x**

4.8.3. Talent placing the gel on a millimeter paper or measuring the gel with a caliper.

5. Mounting and Imaging the Gel

Demonstrator: Léo Krüttli

5.1. Coat clean coverslips with approximately 200 milliliters of poly-lysine solution **[1]**. Incubate the coverslips for 1 hour at room temperature **[2]**, then wash them three times with double-distilled water to remove any excess poly-lysine **[3]**. After the coverslips are dry, store them at 4 degrees Celsius until further use **[4]**.

5.1.1. Talent pipetting poly-lysine solution over clean coverslips.

5.1.2. Talent placing the coverslips on the bench for incubation at room temperature.

5.1.3. Talent placing the coverslips in a dish filled with double-distilled water.

5.1.4. Talent placing the dried coverslips in a refrigerator.

~~5.2. Next, place the expanded gels onto non-coated coverslips situated in the imaging chamber **[1.** Perform fluorescence microscopy to check and confirm the orientation of the samples **[2]**.~~
NOTE:Step deleted by authors

~~5.2.1. Talent carefully transferring gel samples onto plain coverslips inside the imaging chamber.~~

~~5.2.2. Shot of talent operating the fluorescence microscope.~~

5.3. Place the gel properly orientated with the cells facing up onto lint-free paper and allow them to dry to eliminate excess water **[1]**.

5.3.1. Talent placing oriented gels onto lint-free paper and patting them dry.

~~5.4.~~ Finally, mount the dried gels with the cells facing down onto poly-D-lysine-coated coverslips **[1]**. ~~and observe the mounted samples using an inverted microscope **[2]**.~~

5.4.1. Talent carefully mounting the dry gel onto poly-D-lysine-coated coverslip.

~~5.4.2. Talent placing the sample in an inverted microscope.~~
NOTE:Shot deleted by authors

Results

6. Results

6.1. Cryo-fixation preserved the mitochondrial network in RPE1 cells more effectively than PFA fixation, as shown by NHS-ester and ATP5a staining **[1]**, and allowed resolution of mitochondrial cristae, which were not visible with PFA fixation **[2]**.

6.1.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the NHS-ester and ATP5a Images*.

6.1.2. LAB MEDIA: Figure 3B. *Video editor: Zoom in on the red-arrowed mitochondrial cristae in 3B"*.

6.2. Cryo-fixation resulted in better preservation of dynamic microtubules, including cytoplasmic and astral microtubules in mitotic RPE1 cells **[1]**, compared to PFA fixation **[2]**.

6.2.1. LAB MEDIA: Figure 3D, 3E. *Video editor: Highlight the area pointed by red-arrows in D*.

6.2.2. LAB MEDIA: Figure 3D, 3E. *Video editor: Highlight the area pointed by red-arrows in E*.

6.3. In *Trypanosoma brucei*, cryo-fixation better preserved the architecture of the mitochondrion and general cellular structure **[1]** compared to PFA fixation, as visualized with TDH and NHS-ester staining **[2]**. Additionally, cryo-fixation preserved the endoplasmic reticulum in them better than PFA fixation, based on BiP *(B-I-P)* staining **[3]**.

6.3.1. LAB MEDIA: Figure 4A, 4B, 4C, 4D. *Video editor: Highlight yellow TDH image and NHS ester in A*

6.3.2. LAB MEDIA: Figure 4A, 4B, 4C, 4D. *Video editor: Highlight yellow TDH image and NHS ester in C*

6.3.3. LAB MEDIA: Figure 4E, 4F. *Video editor: Highlight the BiP image in E*

6.3.4. LAB MEDIA: Figure 4E, 4F. *Video editor: Highlight the BiP image in F*

6.4. Cracks were observed in cryo-fixed RPE1 cells, but these did not disrupt the ultrastructure of organelles such as mitochondria or microtubules **[1]**.

6.4.1. LAB MEDIA: Figure 5A. *Video editor: Show the cracks (indicated by WHITE arrowheads).*

6.5. Poor cryo-fixation was indicated by bubble-like structures and wavy microtubules, accompanied by loss of membranous organelle integrity **[1]**.

6.5.1. LAB MEDIA: Figure 5B. *Video editor: Highlight the bubble-like formations indicated by white arrowheads*.

6.6. Cryo-fixation quality was dependent on the clarity of sodium acrylate solutions, with usable solutions appearing colourless or slightly yellow and translucent **[1]**, while unusable ones appeared cloudy and orange **[2]**.

6.6.1. LAB MEDIA: Figure 5C. *Video editor: highlight the tubes 1 and 2*

6.6.2. LAB MEDIA: Figure 5C. *Video editor: highlight the tube 3*

Pronunciation Guide:

1. microtubules

Pronunciation link: https://www.merriam-webster.com/dictionary/microtubules YouTube+10How To Pronounce+10How To Pronounce+10

IPA: /ˌmaɪ.kroʊˈtuː.bjuːlz/

Phonetic Spelling: my-kroh-TOO-byools

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2. tubulin

Pronunciation link: https://www.merriam-webster.com/dictionary/tubulin Merriam-Webster

IPA: /ˈtuː.bə.lɪn/

Phonetic Spelling: TOO-buh-lin

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3. Trypanosoma brucei

Pronunciation link: https://www.definitions.net/pronounce/trypanosoma%20brucei Definitions

IPA: /ˌtraɪpənoʊˈsoʊmə ˈbruːsaɪ/

Phonetic Spelling: try-puh-noh-SOH-muh BROO-sigh

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4. ciliogenesis

Pronunciation link: No confirmed link found

IPA: /ˌsɪ.li.oʊˈdʒɛn.ə.sɪs/

Phonetic Spelling: sih-lee-oh-JEN-uh-sis

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5. morphogenesis

Pronunciation link: No confirmed link found

IPA: /ˌmɔːr.foʊ.dʒəˈnɛs.ɪs/

Phonetic Spelling: mor-foh-jen-ESS-is

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6. centriole

Pronunciation link: No confirmed link found

IPA: /ˈsɛn.tri.oʊl/

Phonetic Spelling: SEN-tree-ohl

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7. ultrastructure

Pronunciation link: No confirmed link found
IPA: /ˈʌl.trəˌstrʌk.tʃər/
Phonetic Spelling: UL-truh-STRUK-chur

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8. super-resolution

Pronunciation link: No confirmed link found
IPA: /ˈsuː.pər ˌrɛzəˈluː.ʃən/
Phonetic Spelling: SOO-per rez-uh-LOO-shun

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9. cryo-fixation

Pronunciation link: No confirmed link found
IPA: /ˈkraɪ.oʊ fɪkˈseɪʃən/
Phonetic Spelling: KRY-oh fik-SAY-shun

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10. Vitrobot

Pronunciation link: No confirmed link found
IPA: /ˈvaɪ.troʊ.bɑːt/
Phonetic Spelling: VY-troh-bot

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11. ethane

Pronunciation link: No confirmed link found
IPA: /ˈiːθeɪn/
Phonetic Spelling: EE-thayn

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12. acrylamide

Pronunciation link: No confirmed link found
IPA: /əˈkrɪl.əˌmaɪd/
Phonetic Spelling: uh-KRIL-uh-myd

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13. formaldehyde

Pronunciation link: No confirmed link found
IPA: /ˌfɔːr.məˈlɛ.haɪd/
Phonetic Spelling: for-muh-LE-hyd

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14. TEMED

Pronunciation link: No confirmed link found
IPA: /ˈtɛmɛd/
Phonetic Spelling: TEM-ed

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15. microcentrifuge

Pronunciation link: No confirmed link found
IPA: /ˌmaɪ.kroʊˈsɛn.trəˌfjuːdʒ/
Phonetic Spelling: my-kroh-SEN-truh-fyoohj

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16. poly-lysine / poly-D-lysine

Pronunciation link: No confirmed link found
IPA: /ˌpɑː.liˈlaɪ.saɪn/
Phonetic Spelling: PAH-lee-LY-syne

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17. RPE1

Pronunciation link: No confirmed link found
IPA: /ɑːr piː iː wʌn/
Phonetic Spelling: R-P-E-one

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18. BiP

Pronunciation link: No confirmed link found
IPA: /bɪp/
Phonetic Spelling: bip

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19. sodium acrylate

Pronunciation link: No confirmed link found
IPA: /ˈsoʊ.di.əm əˈkrɪl.eɪt/
Phonetic Spelling: SOH-dee-um uh-KRIL-ayt