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Title: Cryo-structured Illumination Microscopic Data Collection from Cryogenically Preserved Cells

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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? **Yes**

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: 30
Number of Shots: 56

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Nina Vyas:** This method makes cryo-imaging possible at super resolution on whole unstained biological cells to precisely identify cellular structures. It can also be used in conjunction with other imaging techniques as part of a correlative imaging workflow.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll4.1*

1.2. **Nina Vyas:** The main advantage of this technique is that super resolution imaging can be rapidly done in cryo conditions using conventional fluorophores, with relatively low light doses.

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

OPTIONAL:

1.3. **Chidinma Okolo:** CryoSIM is a powerful tool that could provide insight towards understanding cellular ultrastructure dynamics in response to external or internal cues. Its application includes quality control and post-marketing surveillance vaccine production and roll-out, antibody engineering and optimization, and nanoparticles characterisation.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 6.2.1*

1.4. **Nina Perry:** Maneuvering samples within the cryo-stage takes practice to ensure safety of the grid. It's also best to familiarize with the controls in the cockpit window before data collection.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.3.1*

Protocol

2. Preparation of the Cryo-Stage

2.1. To begin, remove the lid from the external dewar of the cryo-stage **[1]** and pour filtered liquid nitrogen until approximately a quarter full. Wait until initial boiling subsides before pouring more and fill the vessel to about two-third full **[2].** Replace the lid carefully, pointing the nozzle away from the handler as the liquid nitrogen boils out **[3]**.

2.1.1. Talent removing the lid from the dewar of cryo-stage

2.1.2. Talent pouring liquid nitrogen

2.1.3. Talent replacing the lid, pointing the nozzle away

2.2. Once the liquid nitrogen has stopped coming out of the outlet, place the outlet pipe over the stage dewar on the cryo-stage **[1]**.

2.2.1. Talent placing the outlet pipe over the stage drawer

2.3. Plug-in the power source, connect the USB cable, and plug in the external dewar to the stage **[1]**.

2.3.1. Talent plugging in the power source, the USB cable and the external dewar to the stage

2.4. After delivery of liquid nitrogen, press the release button on the cryo-stage to allow it to enter the sample chamber and wait for 30 to 45 minutes for the system to cool and stabilize before commencing with image acquisition **[1]**.

2.4.1. Talent pressing the release button

3. Transfer of the Sample Storage Box into the Cryo-Stage

3.1. Use the hex key on the cassette tool to open the two plates of the sample transfer cassette. Open the plates wide enough to drop the grid between the two plates, but do not open to the maximum position **[1]**.

3.1.1. Talent using the hex key to open the plates of sample transfer cassette *Videographer: This step is important!*

3.2. Use long forceps to lift the sample grid box out of the liquid nitrogen **[1]**, turn it so that the notch aligns with the storage position inside the stage and place it onto the stage **[2]**. Use the appropriate device to open the storage box lid to the correct sample position **[3]**.

3.2.1. Talent lifting the sample grid box out of liquid nitrogen

3.2.2. Talent turning the box to suitable notch alignment *Videographer: This step is important!*

3.2.3. Talent unscrewing to open the storage box *Videographer: This step is important!* NOTE: From 3.2.3 to 3.4.2 the shots are combined as one

3.3. Using inverted forceps, remove the TEM grid from the sample holder **[1]**, immerse it inside the liquid nitrogen **[2]** and ensure the carbon film side is placed so that it will ultimately be facing the objective on the sample bridge **[2a],** and drop it into position in the sample transfer cassette **[3]**. Close the sample cartridge using the hex key on the cassette tool **[4]**.

3.3.1. Talent removing TEM grid from holder *Videographer: This step is important!*

3.3.2. Talent immersing TEM grid in liquid nitrogen *Videographer: This step is difficult important!*

3.3.2a Added shot: Talent rotating the grid to the correct orientation inside the liquid nitrogen

3.3.3. Talent dropping TEM grid *Videographer: This step is difficult and important!*

3.3.4. Talent closing sample cartridge using hex key *Videographer: This step is important!* NOTE: second half of action, removal of cartridge is moved to new shot 3.4.3

3.4. Use the magnet point on the cassette tool to lift and mount the cartridge containing the grid onto the sample bridge **[1]**. Keep it immersed or close to the liquid nitrogen and in proper orientation. Place the cassette flat within the positioning pins of the bridge and gently nudge it to ensure it is fixed **[2]**.

3.4.1. Talent lifting and mounting the cartridge grid on sample bridge *Videographer: This step is important!*

3.4.2. Talent placing the cassette flat with positioning pins and nudging it to check if it's fixed *Videographer: This step is important!*

3.5. Close and remove the storage box along with any remaining samples **[4]**.

3.4.3 Added shot: Talent removing the grid box NOTE: contains second half of action from shot 3.3.4

4. Stage Docking and Focusing

4.1. Move the cryo-stage lid opening to the imaging position and turn off the sample chamber light **[1]**. Slide the stage towards the optics to align it under the objective lens **[2]**, then gently drop the objective into position using the lever, ensuring that it rests within the lid of the cryo-stage but does not touch it **[3]**.

4.1.1. Talent moving the cryo-stage lid opening to the imaging position

4.1.2. Talent sliding the stage towards the optics

4.1.3. Talent dropping the objective into position within lid of cryo-stage

4.2. Cover the stage and optics with an opaque black curtain **[1]**, then start the control software Cockpit on the cryoSIM PC **[2]**.

4.2.1. Talent covering the stage with black curtain

4.2.2. WIDE: Talent sitting and opening the software on the computer

4.3. Click on the **readout mode** button for each camera and set it to **CONV 3 Megahertz**. Check that the temperature of each camera is -80 degrees Celsius and that the camera fan is off **[1]**.

4.3.1. SCREEN: 62274_screenshot1.mp4. 0:02-0:17

4.4. Turn on the **reflected** camera **[1]**. Under **Lights**, choose **ambient** and under **linkam**, click on **condenser [2-TXT]**, then click on the **Video mode** button **[3]**.

4.4.1. SCREEN: 62274_screenshot2.mp4. 0:00-0:05

4.4.2. SCREEN: 62274_screenshot2.mp4. 0:06-0:14 **TEXT: exposure 20 ms**

4.4.3. SCREEN: 62274_screenshot2.mp4. 0:16-0:20

4.5. In the **Mosaic view** window, zoom out to see the grid outline **[1]**. Click on **Find stage** if it cannot be seen and center the grid by double-left-clicking in the middle of the circle **[2]**.

4.5.1. SCREEN: 62274_screenshot3.mp4. 0:00-0:12

4.5.2. SCREEN: 62274_screenshot3.mp4 0:15-0:19

4.6. Use the **up** and **down** keys to focus the sample until the grid support film or any other relevant feature is in focus **[1]**. Use the **9** and **3** keys on the numerical pad to change the z-step, setting it to 100 micrometers for initial focusing **[2]**.

4.6.1. SCREEN: 62274_screenshot3.mp.0:20-0:24

4.6.2. SCREEN: 62274_screenshot3.mp4.0:27-0:55 *Video editor: Speed up the alignment running*

5. Brightfield Mosaic Acquisition and Identification of Areas of Interest

5.1. Once the stage is centered, turn off **video mode**. Collect a visible light mosaic by clicking on **Run mosaic** in the **Mosaic view** to produce tiles of visible light images that spiral outward from the center **[1]**. Save the view by clicking on **Save mosaic [2]**.

5.1.1. SCREEN: 62274_screenshot4.mp4. 0:00 – 0:10, then 5:13 – 5:23. *Video editor: Speed up the alignment running*

5.1.2. SCREEN: 62274_screenshot4.mp4.5:24-5:31

5.2. Inspect the brightfield mosaic alongside any previous fluorescence "map" images by turning off the **ambient light** and **condenser** as well as the **video mode [1]**. Turn on the required excitation laser and choose the corresponding **camera** and **filter**, initially at 50 milliwatt for a 50-millisecond exposure time **[2]**.

5.2.1. SCREEN: 62274_screenshot5.mp4.0:00-0:06

5.2.2. SCREEN: 62274_screenshot5.mp4.0:06-0:30

5.3. Press **0** to snap an image and **star** to auto-contrast. Alternatively, manually adjust the contrast by using the slider at the bottom of the image **[1]**.

5.3.1. SCREEN: 62274_screenshot5.mp4.0:32-0:51

5.4. Once biologically interesting cells with suitable fluorescence have been found, mark their positions using the **Mark site** button in **Mosaic view [1]**.

5.4.1. SCREEN: 62274_screenshot5.mp4.0:53-0:57

5.5. Continue marking all potential sites before beginning image acquisition. Re-save the mosaic with the marked sites by clicking on **Save sites to file [1]**.

5.5.1. SCREEN: 62274_screenshot5.mp4.1:29-1:37

5.6. To stitch the mosaic image using the StitchM software, drag and drop the **.txt mosaic** file into the StitchM file with the extension **.bat** and save the combined tiff image of the mosaic tiles in the same folder. To save an image with the marked sites, drag and drop the **mosaic.txt** file and the **markers.txt** file into the icon at the same time **[1-TXT]**.

5.6.1. SCREEN: 62274_screenshot6.mp4.0:00-0:32 **TEXT: Developed in-house at beamline B24**

6. Data Collection

6.1. Set the laser exposure time based on the counts in the dynamic range in the fluorescence image at the bottom of the camera view window. Choose which filter to apply and optimize the settings for each wavelength of excitation light to be used, turning each laser on separately **[1]**.

6.1.1. SCREEN: 62274_screenshot7.mp4.0:00-0:30

6.2. Click on both **cameras** to turn them on. Return to one of the marked sites and focus on the desired depth again **[1]**.

6.2.1. SCREEN: 62274_screenshot7.mp4.0:35-0:57

6.3. Once in focus in an area of interest, move out of focus using the up-arrow key in the **XY** window on the **Macro Stage** to choose the height of the z stack to acquire, and click on **Save top [1]**. Move out of focus using the down arrow key, click on **Save bottom**, then on **Go to the centre**. Verify that the image is still in focus **[2]**.

6.3.1. SCREEN: 62274_screenshot7.mp4.1:25-1:35

6.3.2. SCREEN: 62274_screenshot7.mp4.1:36-1:45

6.4. In the **Cockpit** window, select **Single-site experiment**. From the dropdown list, select **Structured Illumination [1]**. Alter the **Stack height** so that it equals the z-height plus 1 micrometer **[2]**.

6.4.1. SCREEN: 62274_screenshot7.mp4.1:52-1:58

6.4.2. SCREEN: 62274_screenshot7.mp4.1:59-2:05

6.5. Enter the exposure times in milliseconds for the 405 and 488-nanometer lasers in the upper row for the reflected camera and the exposure times for the 561 and 647-nanometer lasers in the lower row for the transmitted camera **[1]**.

6.5.1. SCREEN: 62274_screenshot7.mp4.2:06-2:12

6.6. Input a file name and click on **Update** to produce a new file containing the date and time without overwriting previous files. Then, click on **Start [1]**.

6.6.1. SCREEN: 62274_screenshot7.mp4.2:13-3:05 *Video editor: Speed up the alignment running*

6.7. If the liquid nitrogen dewar refills the cryo-stage during image acquisition, abort the process by clicking on the **Abort** button in the **Cockpit** software **[1]**.

6.7.1. SCREEN: 62274_screenshot7.mp4.3:55-4:05

6.8. At each position, collect a z stack using visible light by Switching off the lasers, and switching on the **Ambient light** and **condenser [1]**. Under **Single-site experiment**, select **Z-stack** and set the **Ambient light** to 20-millisecond exposure, maintaining the z height. Repeat this process for all marked sites **[2]**.

6.8.1. SCREEN: 62274_screenshot8.mp4.0:00-0:17

6.8.2. SCREEN: 62274_screenshot8.mp4.0:00-0:40

7. After Imaging

7.1. After imaging is finished, undock the stage, and remove all samples. Turn off the sample chamber light **[1]**. Unplug the external dewar and decant any remaining liquid nitrogen into another cryo-compatible container, allowing the dewar to safely return to a normal temperature **[2]**.

7.1.1. Talent undocking the stage and removing all the samples

7.1.2. Talent decanting the remaining liquid nitrogen into container

7.2. Wait until the option to **bake-out** the cryo-stage display becomes available after no more liquid nitrogen remains in the stage dewar. Press the **bake-out** button to enter

the heating mode **[1].** Put the lid plug on the cryo-stage **[2]**. NOTE: Script change: Shot positions 7.2.1 and 7.2.2 and accordingly the voice overs are now exchanged

7.2.1. Option to bake-out is available and talent pressing the bake-out button to enter the heating mode

7.2.2. Talent putting the lid- plug on the cryo-stage

Results

8. Results: Cryo-structured Illumination Microscopic Imaging

8.1. The resolution in cryoSIM is significantly higher than that in standard epifluorescence microscopy **[1]**. The fluorescent "map" from a conventional epifluorescence microscope can be used to locate areas of interest for imaging **[2]** and the corresponding cryoSIM-image can be obtained from a location on the grid **[3]**.

8.1.1. LAB MEDIA: Figure 6

8.1.2. LAB MEDIA: Figure 6. *Video Editor: Emphasize A and B.*

8.1.3. LAB MEDIA: Figure 6. *Video Editor: Emphasize C.*

8.2. A sample containing U2OS cells was stained with a mixture of green microtubule cytoskeleton dye and red mitochondria dye, resulting in the staining of the microtubule component of the cytoskeleton and the mitochondria **[1]**.

8.2.1. LAB MEDIA: Figure 5C, 5D

8.3. Subsequent imaging showed the localization of mitochondria within the cell as well as the arrangement of the microtubules, highlighting the structural framework that they provide to the cell and the assembly of the cytoskeleton around organelles **[1]**.

8.3.1. LAB MEDIA: Figure 5C, 5D

8.4. The SIM reconstruction process can produce artifacts, which can be identified using SIM-Check, a free ImageJ plugin. This modulation contrast map generated using SIM-Check shows areas of low modulation contrast within the nucleus area, indicating that this region is going to be more susceptible to reconstruction artifacts **[1]**.

8.4.1. LAB MEDIA: Figure 5D, 5E

Conclusion

9. Conclusion Interview Statements

9.1. **Nina Perry:** When attempting this protocol, ensure that the samples stays submerged or close to liquid nitrogen at all times to avoid devitrification. Also, pay attention to exposure times and counts in the dynamic range, since this affects the quality of the data and avoids laser damage to the sample.

9.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2.3, 3.3.2, 3.4.2 and 7.1.1*

9.2. **Nina Vyas:** Following cryoSIM imaging, the same samples can be imaged with other modalities such as cryo soft x-ray tomography, which does not require cells to be stained. By combining cryoSIM images with images giving structural information about the cells, we can answer additional key questions on cell ultrastructure and function.

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