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Title: In-Nucleus Hi-C in Drosophila Cells

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Author Questionnaire

1. **Microscopy**: Does your protocol demonstrate the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **N**

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 the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

☒ Interviewees self-record interview statements outside of the filming date. JoVE can provide support for this option.

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **52**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Ayerim Esquivel**: The in nucleus Hi-C protocol allows the exploration of chromosomal conformations at different resolutions for the characterization of chromatin loops, domains, and compartments organizing the genome **[1]**.

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

REQUIRED:

1.2. **Mayra Furlan**: This protocol provides high-resolution profiling of genomic interactions at different scales, yielding a more consistent coverage over the full range of genomic distances and data with less technical noise **[1]**.

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

OPTIONAL:

1.3. **Rosario Perez**: Combining this protocol with genetic editing is a powerful strategy for testing the structural function of genetic elements. It can also be applied to the characterization of the structural variations that lead to disease **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

OPTIONAL:

1.4. **Rosario Perez**: The in nucleus Hi-C protocol can be applied to explore the genome organization of any eukaryotic genome **[1]**.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

OPTIONAL:

1.5. **Ayerim Esquivel**: During SDS and triton treatment, disaggregate any nuclear clumps by pipetting, as aggregates interfere with the enzymatic reaction efficiency, and be sure to perform the appropriate quality control measures prior to sequencing **[1]**.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

Protocol

2. Schneider´s Line 2 Plus (S2R+) Drosophila Cell Fixation

2.1. Begin by adding methanol-free formaldehyde to 1 x 10^7 Schneider's line 2 plus *Drosophila* cells in 17.5 milliliters of Schneider medium supplemented with 10% FBS to a final concentration of 2% **[1-TXT]**.

2.1.1. WIDE: Talent mixing formaldehyde into cells, with formaldehyde container visible in frame *Videographer: Important step* **TEXT: See text for all medium, buffer, and solution preparation details**

2.2. Incubate the cells for 10 minutes at room temperature, with mixing every 60 seconds **[1]**, and add glycine to a final concentration of 0.125-molar with mixing **[2]**.

2.2.1. Talent setting timer, with cells visible in frame

2.2.2. Talent adding glycine to cells, with glycine container visible in frame

2.3. After 5 minutes at room temperature and 15 minutes on ice, collect the cells by centrifugation **[1-TXT]** and carefully resuspend the pellet in 25 milliliters of cold PBS **[2]**.

2.3.1. Talent adding tube(s) to centrifuge **TEXT: 5 min, 400 x g, RT**

2.3.2. Shot of pellet if visible, then PBS being added to tube, with PBS container visible in frame

2.4. Then collect the cells with a second centrifugation **[1-TXT]**.

2.4.1. Talent adding tube to centrifuge **TEXT: 10 min, 400 x g, 4 °C**

3. Lysis

3.1. At the end of the centrifugation, resuspend the cells in 1 milliliter of ice-cold lysis buffer for counting ~~**[1]**~~ and adjust the cells to a 1 x 10^6 cells/milliliter concentration **[1]**.

3.1.1. WIDE: Talent adding buffer to tube, with buffer container and cell counter visible in frame

~~3.1.2. Talent adding buffer to tube, with buffer container visible in frame~~

3.2. Incubate the cells on ice for 30 minutes, inverting the tube every 2 minutes to mix **[1]**, and collect the cells with another centrifugation **[2-TXT]**. Resuspend the pellet in 1 milliliter of fresh ice-cold lysis buffer **[3]**.

3.2.1. Talent placing tube on ice

3.2.2. Talent placing tube(s) into centrifuge **TEXT: 5 min, 300 x g, 4 °C**

3.2.3. Shot of pellet if visible, then buffer being added to tube

3.3. After transfer to a microcentrifuge tube containing milliliter of 1.25x restriction buffer **[1]**, centrifuge to collect the lysate **[2]**.

3.3.1. Talent adding pellet to microcentrifuge tube, with buffer container visible in frame NOTE: Slated as 3.3.1A

3.3.2. Talent placing tube into centrifuge NOTE: Slated as 3.3.1

3.4. Resuspend the pellet in 360 microliters of fresh restriction buffer and 11 microliters of 10% SDS (S-D-S) with careful pipetting **[1]** and incubate for 45 minutes at 37 degrees Celsius and 700-950 revolutions per minute with occasional pipetting **[2]**.

3.4.1. Shot of pellet if visible, then buffer and/or SDS being added to tube, with buffer and SDS containers visible in frame *Videographer: Important/difficult step* NOTE: 3.4.1A: Buffer is already added, shot of pellet, 3.41B: rest of the shot of 3.4.1

3.4.2. Talent placing tube at 37 °C *Videographer: Important step*

3.5. At the end of the incubation, stop the permeabilization with 75 microliters of non-ionic surfactant **[1]** and return the tube to the incubator for an additional 45 minutes with shaking at 950 revolutions per minute with occasional pipetting **[2]**.

3.5.1. Talent adding surfactant to tube, with surfactant container visible in frame

3.5.2. Shot of tube on shaker

4. Enzymatic Digestion, DNA End Biotinylation, and Ligation

4.1. For chromatin digestion, add 200 units of Mbo (M-B-O) one to the tube for a 4-16-hour incubation at 37 degrees Celsius with rotation **[1]**.

4.1.1. WIDE: Talent adding Mbo I to tube, with Mbo I container visible in frame

4.2. At the end of the incubation, inactivate the enzyme with a 20-minute incubation at 60 degrees Celsius **[1]**.

4.2.1. Talent placing tube at 60 °C

4.3. To fill in the restriction fragment overhangs and to label the DNA ends with biotin, add 1.5 microliters each of 10-millimolar dCTP (D-C-T-P), dGTP, and dTTP, 20 microliters of 0.4-millimolar biotin dATP, 17.5 microliters of Tris low-EDTA buffer, and 10 microliters of 5 units/microliter of DNA polymerase one large fragment to the tube with careful mixing **[1]**.

4.3.1. Talent adding reagent(s) to tube, with reagent containers visible in frame
Videographer: Important/difficult step

4.4. Incubate the reaction for 75 minutes at 37 degrees Celsius with shaking at 700 revolutions per minute every 10 seconds for 30 seconds **[1]**.

4.4.1. Tube on rotator NOTE: Preferably include the stop of shaking.

4.5. At the end of the incubation, transfer the chromatin mixture to a new 1-milliliter tube containing 100 microliters of ligation mix with thorough, gentle mixing **[1]**, and incubate overnight at 16 degrees Celsius **[2]**.

4.5.1. Talent adding mixture to tube, with mix container visible in frame

4.5.2. Talent placing tube at 16 °C

5. Crosslink Reversal and DNA Purification

5.1. To degrade the sample proteins, add 50 microliters of 10 milligram/milliliter Proteinase K to the tube for a 2-hour incubation at 37 degrees Celsius **[1]**.

5.1.1. WIDE: Talent adding proteinase K to tube, with proteinase K container visible in frame

5.2. At the end of the incubation, increase the temperature to 65 degrees Celsius overnight to reverse crosslink the sample **[1]**. The next morning, degrade the RNA with 10 microliters of 10 milligram-milliliter RNase A **[2]**.

5.2.1. Talent increasing temperature

5.2.2. Talent adding RNase to tube, with RNase container visible in frame

5.3. After 1 hour at 37 degrees Celsius, add 1 volume of phenol-chloroform to the tube **[1]** and mix thoroughly by inversion to obtain a homogeneous white phase **[2]**.

5.3.1. Talent adding phenol-chloroform to the tube

5.3.2. Tube being inverted/white phase forming

5.4. After precipitating the DNA according to standard protocols **[1]**, quantify the DNA using a fluorogenic dye that binds selectively to DNA and a fluorometer according to the manufacturer's instructions **[2]**.

5.4.1. Talent adding ethyl alcohol to tube, with ethyl alcohol container visible in frame

5.4.2. Talent adding dye to tube, with dye and fluorometer visible in frame as possible

6. Hi-C Template Quality Evaluation

6.1. To purify the DNA from undigested and digested aliquots, load 100 nanograms of undigested, digested, and ligated samples onto a 1.5% agarose gel **[1]** and look for a smear centered around 500 base pairs in the digested sample **[2]** versus a high molecular weight band for the ligated sample **[3]**.

6.1.1. WIDE: Talent loading sample(s) onto gel NOTE: Please do not use the first two injections into gel, as they were not perfectly executed

6.1.2. LAB MEDIA: Figure 1B *Video Editor: please emphasize smeared band in Hi-C Mbo I D lanes*

6.1.3. LAB MEDIA: Figure 1B *Video Editor: please emphasize high weight bands in Hi-C Mbo I L lanes*

6.2. To verify the Hi-C marking and ligation efficiency by amplification and digestion of a known interaction, after PCR, digest the products with Mbo one, Cla (C-L-A) one, or both **[1]** and run the samples on a new 1.5-2% gel to allow estimation of the relative number of 3C and Hi-C ligation junctions **[2]**.

6.2.1. Talent adding enzyme to sample(s), with enzyme containers visible in frame

6.2.2. Talent loading sample(s) onto gel

7. Biotin Removal/End Repair and Size Selection

7.1. After sonicating the sample to obtain 200-500 base pair DNA fragments, transfer 130 microliters of each sample into new microcentrifuge tubes containing 16 microliters of 10x ligation buffer **[1]**, 2 microliters of 10-millimolar dATP, 5 microliters of T4 DNA Polymerase, and 7 microliters of double-distilled water for a 30-minute incubation at 20 degrees Celsius **[2]**.

7.1.1. WIDE: Talent adding buffer and/or reagents to tube, with buffer and reagent containers visible in frame

7.1.2. Talent placing tube(s) at 20 °C

7.2. At the end of the incubation, add 5 microliters of 10-millimolar dNTPs, 4 microliters of 10x ligation buffer, 5 microliters of T4 polynucleotide kinase, 1 microliter of DNA polymerase one large fragment, and 25 microliters of double-distilled water to the tubes for a second 30-minute incubation at 20 degrees Celsius **[1]**.

7.2.1. Talent adding buffer and/or reagents to tube, with buffer and reagent containers visible in frame

7.3. To select fragments mostly in the 250-550 base pair size range, perform sequential solid phase reversible immobilization size selection with 0.6x then 0.9x according to the manufacturer's instructions **[1]** and elute the DNA with 100 microliters of Tris-low EDTA **[2]**.

7.3.1. Talent mixing beads with sample/placing sample onto magnet NOTE: Preferably crossfade take A to take B, normally takes 1 to 1 and a half minutes

7.3.2. Talent removing supernatant/adding TLE

8. Biotin Pulldown/A-Tailing/Adapter Ligation

8.1. For biotin pulldown, for each library, wash 150 microliters of streptavidin-linked magnetic beads two times with 400 microliters of 1x Tween buffer **[1]** and rotate the sample for 3 minutes on a rotating wheel **[2]**.

8.1.1. WIDE: Talent adding buffer to tube, with buffer container visible in frame

8.1.2. Talent placing tube onto wheel

8.2. At the end of the incubation, resuspend the beads in 300 microliters of 2x no-Tween buffer **[1]** and mix with 300 microliters of Hi-C material for a 30-minute rotation on a rotating wheel **[2]**.

8.2.1. Talent adding NTB to beads, with NTB container visible in frame

8.2.2. Talent add Hi-C container to tube, with wheel visible in frame if possible

8.3. At the end of the incubation, wash the beads with 400 microliters of 0.5x Tween buffer **[1]** for a 3-minute incubation at 55 degrees Celsius and 750 revolutions per minute **[2]** followed by two washes in 200 microliters of 1x restriction buffer per wash **[3]**.

8.3.1. Talent adding buffer to tube, with buffer container visible in frame

8.3.2. Talent placing tube onto rotator

8.3.3. Talent adding buffer to tube, with buffer container visible in frame

8.4. After the second wash, resuspend the beads in 100 microliters of dATP tailing mix for a 30-minute incubation at 37 degrees Celsius **[1]**.

8.4.1. Talent adding dATP mix to tube, with dATP mix container visible in frame

8.5. At the end of the incubation, resuspend the beads in 50 microliters of 1x ligation buffer **[1]** and transfer the suspension to a new tube containing 4 microliters pre-annealed pair-end adapters and 2 microliters of T4 ligase **[2]**.

8.5.1. Talent adding buffer to tube, with buffer container visible in frame

8.5.2. Talent adding solution to tube, with pair-end adapter and ligase containers visible in frame

8.6. After 2 hours at room temperature, remove the supernatant **[1]** and wash the beads two times with 400 microliters of Tween buffer **[3]**.

8.6.1. Talent removing supernatant

8.6.2. Talent adding buffer to tube, with buffer container visible in frame

8.7. Then wash the beads one time with 200 microliters of no-Tween buffer **[1]** and one time with 100 microliters of restriction buffer before resuspending the beads in 40 microliters of 1x restriction buffer **[2-TXT]**.

8.7.1. Talent adding NTB to tube, with NTB container visible in frame

8.7.2. Talent adding restriction buffer to tube, with restriction buffer container visible in frame **TEXT: See text for PCR amplification and pair-end sequencing details**

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.1., 3.4., 4.3.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
3.4. Make sure you disaggregate the clumps during SDS plus triton incubation and prior enzymatic digestion
4.3 Biotin labeling and blunt end ligation efficiency needs to be tested performing the controls described. Unsuccessful biotin labeling and ligation will result in a poor quality Hi-C library.

Results

9. Results: Representative In-Nucleus Hi-C Analysis

9.1. A smear of 200-1000 base pairs is observed **[1]** when restriction with Mbo one is successful **[2]**.

9.1.1. LAB MEDIA: Figure 1B
9.1.2. LAB MEDIA: Figure 1B *Video Editor: please emphasize 200-1000 bp smear in Hi-C Mbo I D lanes*

9.2. If the ligation is successful, a high molecular weight band is observed at the top of the gel **[1]**.

9.2.1. LAB MEDIA: Figure 1B *Video Editor: please emphasize band at top of gel in Hi-C Mbo I Lig lanes*

9.3. Digestion efficiency can be also confirmed by quantitative PCR **[1]**. An acceptable digestion efficiency is 80% or higher **[2]**.

9.3.1. LAB MEDIA: Figure 1C
9.3.2. LAB MEDIA: Figure 1C *Video Editor: please added dashed line across graph at 80% to show efficiency cutoff*

9.4. As illustrated, the amplification of a known medium-range interaction in Hi-C ligation products can be estimated by digestion of the PCR product recovered in the amplification **[1]**.

9.4.1. LAB MEDIA: Figure 2A

9.5. A digestion efficiency of more than 70% is recommended **[1]** to avoid having a large proportion of non-useful reads for the libraries after sequencing **[2]**.

9.5.1. LAB MEDIA: Figure 2A gel *Video Editor: please emphasize band(s) in ClaI + MbIL- 3C lane*
9.5.2. LBA MEDIA: Figure 2A gel *Video Editor: please emphasize band(s) in Clai+ MboI- HiC lane*

9.6. The number of PCR cycles for the final amplification **[1]** should be one cycle less than the number of cycles for which the smear is visible **[2]**.

9.6.1. LAB MEDIA: Figure 2B
9.6.2. LAB MEDIA: Figure 2B *Video Editor: please emphasize arrow*

9.7. The level of digestion of the library indicates the abundance of valid Hi-C pairs **[1]** and reflects the proportion of useful reads that will be obtained from the library **[2]**.

9.7.1. LAB MEDIA: Figure 2C *Video Editor: please emphasize ClaI smears*
9.7.2. LAB MEDIA: Figure 2C

9.8. Using the unique valid Hi-C pairs, basic analysis of the pair distribution can be performed **[1]**.

9.8.1. LAB MEDIA: Figures 3B-E

9.9. As observed in this representative example, the *Notch* gene locus can be seen **[1]** along with the architectural proteins **[2]**, domains one and two **[3]**, and histone modifications along the locus **[4]**.

9.9.1. LAB MEDIA: Figure 4A *Video Editor: please emphasize green highlighting in Figure 4A*
9.9.2. LAB MEDIA: Figure 4A *Video Editor: please ChIP-seq tracks underneath the matrix in Figure 4A*
9.9.3. LAB MEDIA: Figure 4A *Video Editor: please emphasize Domain 1 and Domain 2 boxes in Figure 4A*
9.9.4. LAB MEDIA: Figure 4A *Video Editor: please emphasize H3K rows*

9.10. Upon deletion of the region containing both the CTCF (C-T-C-F) and M1BP (M-one-B-P) DNA-binding sites **[1]**, a dramatic change in chromatin contacts can be observed **[2]**.

9.10.1. LAB MEDIA: Figures 4C and 4D *Video Editor: please emphasize 5pN-delta343 graph in Figure 4C*
9.10.2. LAB MEDIA: Figures 4C and 4D *Video Editor: please emphasize 5pN-delta343 graph in Figure 4D*

Conclusion

10. Conclusion Interview Statements

10.1. **Ayerim Esquivel**: After the Hi-C experiment, a capture procedure can be performed to enrich for a specific set of contacts, for example, from promoter regions. This is particularly useful when assessing large genomes **[1]**. *Videographer: Can cut for time*

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

10.2. **Mayra Furlan**: As demonstrated, combining the Hi-C protocol with a genetic edition can be used to assess the structural and regulatory functions of genomic elements **[1]**.

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