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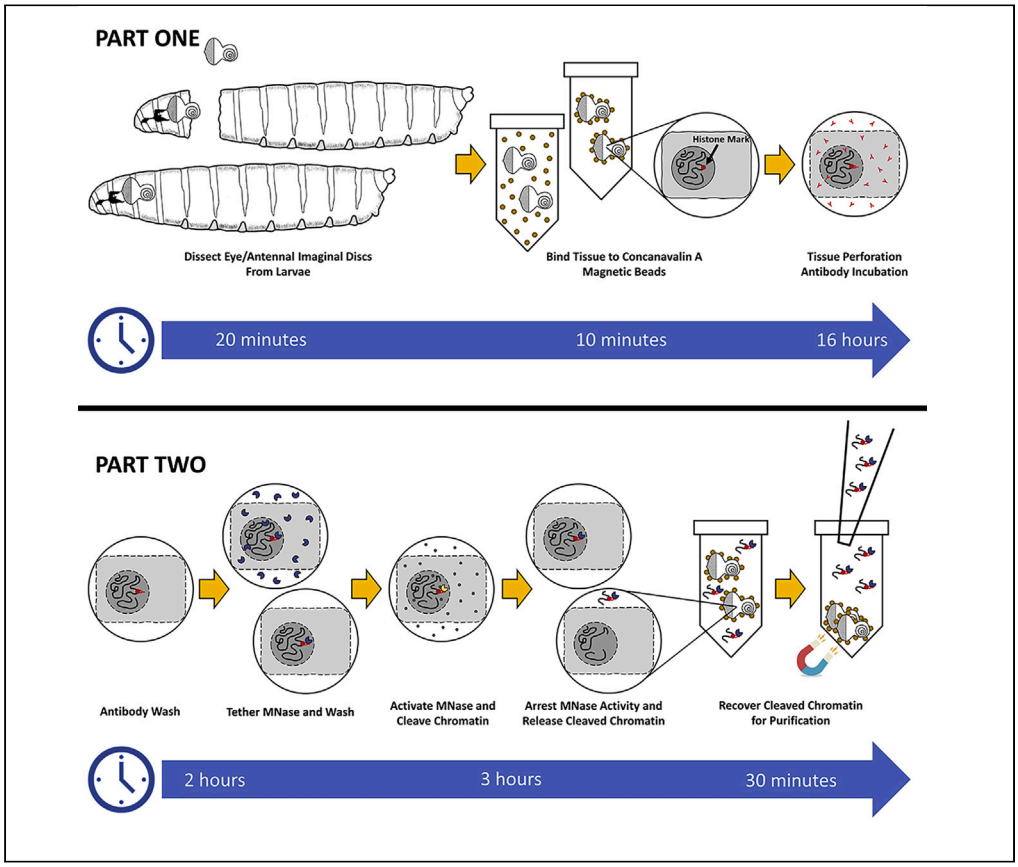
A CUT&RUN protocol to determine patterns of epigenetic marks in imaginal discs of *Drosophila*



Cleavage Under Targets & Release Using Nucleases (CUT&RUN) sequencing is a technique used to study gene regulation. The protocol presented here has been used successfully to identify the pattern of histone modifications within the genome of the eye-antennal disc of the fruit fly, *Drosophila melanogaster*. In its present form, it can be used to analyze genomic features of other imaginal discs. It can be modified for use with other tissues and applications including identifying the pattern of transcription factor occupancy.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

CUT&RUN-based mapping of histone modifications

Optimized for use with imaginal discs from *Drosophila melanogaster*

Purification of Protein A/G-MNase for use with CUT&RUN

Pipeline for bioinformatic analysis of CUT&RUN sequencing data

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A CUT&RUN protocol to determine patterns of epigenetic marks in imaginal discs of *Drosophila*

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SUMMARY

Cleavage Under Targets & Release Using Nucleases (CUT&RUN) sequencing is a technique used to study gene regulation. The protocol presented here has been used successfully to identify the pattern of histone modifications within the genome of the eye-antennal disc of the fruit fly, *Drosophila melanogaster*. In its present form, it can be used to analyze genomic features of other imaginal discs. It can be modified for use with other tissues and applications including identifying the pattern of transcription factor occupancy.

BEFORE YOU BEGIN

The following protocol begins with instructions for producing and purifying the Protein A/G-MNase enzyme in-lab. If a commercially-sourced MNase will be used, then first part of the protocol (the sections labeled "Purification of MNase") can be skipped. The actual CUT&RUN protocol can be found in the sections labelled "CUT&RUN".

This protocol describes the steps required for a single CUT&RUN experiment (one experimental sample and one negative control) from late third larval instar *Drosophila* eye-antennal imaginal discs. Robust results with abundant chromatin marks such as trimethylation of lysine 27 on histone H3 (H3K27me3) are achieved with as few as ten discs. Success with less frequently placed marks such as trimethylation of lysine 4 on histone H3 (H3K4me3) can be attained by increasing the number of eye-antennal imaginal discs. For the H3K4me3 chromatin mark we used thirty discs. Scale all volumes and instructions accordingly based on your needs.

Prepare reagents for the purification of MNase

Timing: 1 day

1. Prepare LB+kanamycin agar plates.
2. Prepare quality DNA mini-prep of the pAG/MNase plasmid from AddGene.

Prepare primary CUT&RUN solutions

Timing: 2 h

3. Prepare digitonin solution.

4. Prepare Binding Buffer.
5. Prepare Wash Buffer.
6. Prepare Stop Buffer.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
JM101 K12 *E. coli* Competent Cells	Agilent	Cat#200234
Antibodies		
Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit mAb (1:50 dilution)	Cell Signaling Technology	Cat#9733
Anti-Trimethyl-Histone H3 (Lys4), Clone 15-10C-E4, Rabbit Monoclonal (1:50 dilution)	Sigma-Aldrich	Cat#05-745R
Normal Rabbit IgG (1:50 dilution)	Cell Signaling Technology	Cat#2729
Chemicals, peptides, and recombinant proteins		
2-Mercaptoethanol	Sigma/Millipore	Cat#3148-25ML
IPTG (isopropyl-β-D-1-thiogalactopyranoside) 100 mM	Sigma/Millipore	Cat#70527-3
Bovine Serum Albumin Standard Ampules, 2 mg/mL	Thermo Fisher	Cat#23209
Coomassie Plus (Bradford) Assay Reagent	Thermo Fisher	Cat#23238
Glycogen	Sigma-Aldrich	Cat#10901393001
Digitonin	Sigma/Millipore	Cat#D141-100MG
cOmplete Protease-Inhibitor Tablets EDTA-free	Roche	Cat#04693132001
Spermidine	Sigma/Millipore	Cat#S0266-1G
BioMag Plus Concanavalin A Beads	Polysciences	Cat#86057-3
RNaseA	Thermo Scientific	Cat#EN0531
Critical commercial assays		
B-PER Complete Bacterial Protein Extraction Reagent	Thermo Fisher	Cat#89821
HisPur Ni-NTA Purification Kit, 1 mL	Thermo Fisher	Cat#88228
Zeba Spin Desalting Columns, 10 mL	Thermo Fisher	Cat#89893
MinElute PCR Purification Kit	Qiagen	Cat#28004
Recombinant DNA		
Plasmid: pAG/MNase	Addgene	Plasmid #123461
Other		
DNA LoBind Tube 1.5 mL	Eppendorf	Cat#022431021
ThermoMixer F1.5	Eppendorf	Cat#5384000020
ThermoTop Lid for ThermoMixer F1.5	Eppendorf	Cat#5308000003
NanoDrop One Microvolume UV-Vis Spectrophotometer	Thermo Fisher	Cat#ND-ONE-W

MATERIALS AND EQUIPMENT

10% 2-mercaptoethanol Solution		
Reagent	Final concentration	Amount
2-mercaptoethanol	10%	100 μL
ddH_2O	N/A	900 μL
Total	**N/A**	**1 mL**

Equilibration Buffer (Ni-NTA Column)		
Reagent	Final concentration	Amount
PBS (10×)	1×	200 μL
Imidazole (2 M)	10 mM	10 μL
ddH_2O	N/A	1.79 mL
Total	**N/A**	**2 mL**

Wash Buffer (Ni-NTA Column)		
Reagent	Final concentration	Amount
PBS (10×)	1×	1 mL
Imidazole (2 M)	25 mM	125 μL
ddH_2O	N/A	8.9 mL
Total	**N/A**	**10 mL**

Elution Buffer (Ni-NTA Column)		
Reagent	Final concentration	Amount
PBS (10×)	1×	200 μL
Imidazole (2 M)	250 mM	250 μL
ddH_2O	N/A	1.55 mL
Total	**N/A**	**2 mL**

Protease Inhibitor Stock Solution		
Reagent	Final concentration	Amount
Roche cOmplete Protease-Inhibitor Tablet EDTA-free	50×	1 tablet
ddH_2O	N/A	1 mL
Store at 4°C for up to 1 week or −20°C for up to 1 month.		

Digitonin Solution		
Reagent	Final concentration	Amount
Digitonin	5%	100 mg
DMSO	N/A	to 2 mL
Total	**N/A**	**2 mL**
Store at −20°C as 1 mL aliquots.		

Binding Buffer		
Reagent	Final concentration	Amount
HEPES (1 M, pH7.9)	20 mM	8 mL
KCl (1 M)	10 mM	4 mL
$CaCl_2$ (1 M)	1 mM	400 μL
$MnCl_2$ (1 M)	1 mM	400 μL
ddH_2O	N/A	387.2 mL
Total	**N/A**	**400 mL**
Store at 4°C for up to 1 year.		

Wash Buffer		
Reagent	Final concentration	Amount
HEPES (1 M, pH7.5)	20 mM	16 mL
NaCl	150 mM	7.01 g
BSA (30%)	0.1%	2.7 mL
ddH_2O	N/A	to 800 mL
Total	**N/A**	**800 mL**
Store at 4°C for up to 1 year.		

STOP Buffer		
Reagent	Final concentration	Amount
NaCl (2.5 M)	340 mM	1.36 mL
EDTA (0.5 M)	20 mM	400 μL
EGTA (0.5 M)	4 mM	80 μL
Glycogen (20 mg/mL)	50 μg/mL	25 μL
ddH_2O	N/A	8.14 mL
Total	**N/A**	**10 mL**
Store at 4°C for up to 1 year.		

100 mM $CaCl_2$		
Reagent	Final concentration	Amount
$CaCl_2 \cdot 2H_2O$	100 mM	1.47 g
ddH_2O	150 mM	to 100 mL
Total	**N/A**	**100 mL**
Store at 4°C for up to 1 week.		

Wash+ Buffer		
Reagent	Final concentration	Amount
Wash Buffer	N/A	19.6 mL
Protease Inhibitor Stock Solution (50×)	1×	400 μL
Spermidine (1 M)	0.5 mM	10 μL
Total	**N/A**	**20 mL**
Store at 4°C for up to 1 week.		

Dig-Wash+ Buffer		
Reagent	Final concentration	Amount
Wash+ Buffer	N/A	11.9 mL
Digitonin Solution (5%)	0.05%	120 μL
Total	**N/A**	**12 mL**
Store at 4°C for up to 1 week.		

Antibody Buffer		
Reagent	Final concentration	Amount
Dig-Wash+ Buffer	N/A	249 μL
EDTA (0.5 M)	2 mM	1 μL
Total	**N/A**	**250 μL**
Store at 4°C for up to 1 week.		

STOP-R Buffer		
Reagent	Final concentration	Amount
STOP Buffer	N/A	248.7 μL
RNaseA Solution (10 mg/mL)	50 μg/mL	1.25 μL
Total	**N/A**	**250 μL**
Store on ice for up to 1 day.		

STEP-BY-STEP METHOD DETAILS

Note: The first 100 steps of this protocol outline the procedure for the purification of the Protein A/G-MNase from bacteria. If a commercially sourced MNase enzyme is the be used, then these steps can be skipped. The actual CUT&RUN protocol begins on step 101.

Purification of MNase: Prepare reagents

Timing: 1 h

Preparation of reagents needed for the transformation of JM101 competent cells with the pA/G-MNase plasmid.

1. Dilute the pAG/MNase mini-prep to 1 ng/μL.
2. Turn on a 42°C water bath and give sufficient time to reach temperature.
3. Turn on a 37°C shaker and give sufficient time to reach temperature.
4. Thaw 100 μL of JM101 competent cells, keep on ice.

Purification of MNase: Transformation

Timing: 2 h with 16 h incubation

Transformation of JM101 competent E. coli cells with pAG/MNase plasmid from Addgene to prepare for bacterial production of the Protein A/G-MNase protein.

5. Add 1.7 μL of 10% 2-mercaptoethanol solution to 100 μL of thawed JM101 competent cells.
6. Mix by gently tapping the tube.
7. Incubate the cells on ice for 10 min, mix by gently tapping the tube every 2 min.
8. Add 1 μL of the 1 ng/μL pAG/MNase mini-prep dilution to the JM101 cells.
9. Mix by gently tapping the tube.
10. Incubate the cells on ice for 30 min.
11. Heat-shock the cells by incubating the tube in the 42°C water bath for 45 s.
12. Return the tube to ice, incubate on ice for 2 min.
13. Add 900 μL of SOC medium to the cells.
14. Place the tube in the 37°C shaker for 1 h with 250 rpm shaking.
15. Pipette 200 μL of the transformed cell mixture onto a LB+kanamycin plate and spread cells.
16. Incubate the plate at 37°C for 16 h.

Purification of MNase: Prepare reagents

Timing: 1 h

Preparation of regents needed for the large bacterial culture.

17. Prepare and autoclave 250 mL of LB medium in a 1 L flask, allow to cool to room temperature.

Purification of MNase: Inoculating the bacterial starter culture

Timing: 2 h with 16 h incubation

Preparing a bacterial starter culture from a colony on the transformation plate.

18. Remove the transformation plate from the incubator.

19. Pipette 5 mL of LB into a test tube.
20. Pipette 5 μL of kanamycin (50 mg/mL) to the LB in the test tube.
21. Pick a single bacterial colony from the transformation plate, transfer to the test tube with the LB.
22. Incubate the test tube at 37°C with 250 rpm shaking for 16 h.

Purification of MNase: Inoculating the large bacterial culture

Timing: 5 h

Using the bacterial starter culture to seed a large-scale culture.

23. Pipette 250 μL of kanamycin (50 mg/mL) into the 250 mL of LB in the flask that cooled overnight.
24. Pipette the entire 5 mL overnight culture of transformed bacteria into the flask.
25. Place the flask in a shaker and incubate at 37°C with 200 rpm shaking until the OD600 of the culture reaches 0.5–0.6 (approximately 4 h).
26. Remove the flask from the shaker. Cool the shaker to room temperature.

Purification of MNase: Induction of protein A/G-MNase production

Timing: 10 min with 16 h incubation

Turning on bacterial protein production of the Protein A/G-MNase by addition of IPTG.

27. Add 1.25 mL of 100 mM IPTG to the flask (0.5 mM final concentration) to induce bacterial protein production.
28. Place the flask in the shaker and incubate at 25°C with 200 rpm shaking for 16 h.

Purification of MNase: Collection of bacterial pellet

Timing: 2 h, 20 min

Collecting bacterial pellet and determining total weight of bacteria.

29. Weigh the empty bottle that will be used to centrifuge the 250 mL bacterial culture and note weight.
30. Transfer the 250 mL bacterial culture into the centrifuge bottle and centrifuge at room temperature for 15 min at 4,000 × *g*.
31. Decant the supernatant from the bacterial pellet.
32. Centrifuge the bottle again at room temperature for 1 min at 4,000 × *g* to collect all liquid at the bottom of the bottle.
33. Using a serological pipette, remove as much liquid as possible from the bacterial pellet.
34. Weigh the bottle again and note the weight of the bacterial pellet.
35. Freeze the bacterial pellet at −20°C for 2 h.

Purification of MNase: Extraction of total protein from bacteria

Timing: 45 min

Extracting total protein from bacterial pellet using B-PER lysing reagent.

36. Calculate the total weight of the bacterial pellet in grams.
37. Multiply this number by 5 to determine the amount of B-PER Complete Bacterial Protein Extraction Reagent you will need (in milliliters) to fully lyse the bacteria.

38. Pipette the calculated amount of B-PER into a 15 mL conical tube and add the required amount of 50× Protease Inhibitor Stock Solution to the B-PER. Mix by serological pipette.
39. Transfer the B-PER solution to the frozen bacterial pellet. Incubate the bottle containing the bacterial pellet and B-PER solution on ice until the pellet has thawed.
40. Gently pipette the B-PER solution in the bottle until the bacterial pellet is completely resuspended.
41. Transfer the resuspended bacterial pellet back into the 15 mL conical tube.
42. Incubate the tube at room temperature for 15 min with gentle rocking.
43. Centrifuge the 15 mL conical tube at 16,000 × *g* at room temperature for 20 min to separate the soluble from insoluble proteins.
44. Transfer the supernatant containing the soluble proteins (including the Protein A/G-MNase) to a clean 15 mL conical tube and store on ice.

Purification of MNase: Purification of the 6×HIS-tagged protein A/G-MNase

Timing: 1 h, 30 min

Using a nickel column to bind and purify the 6×HIS-tagged Protein A/G-MNase from the total soluble bacterial proteins.

45. Prepare the following solutions:
    a. Equilibration Buffer (Ni-NTA column).
    b. Wash Buffer (Ni-NTA column).
    c. Elution Buffer (Ni-NTA column).
46. Remove a Ni-NTA column from the refrigerator and allow to warm to room temperature.
47. Shake the column to thoroughly resuspend the bead slurry.
48. Snap the end off from the column and place the column in a 15 mL conical tube. Loosen the cap on the column.
49. Centrifuge the column/conical tube at 700 × *g* at room temperature for 2 min to remove the storage buffer.
50. Discard the flow-through from the 15 mL conical tube, place column back into conical tube.
51. Add 2 mL of Equilibration Buffer to the column.
52. Centrifuge the column/conical tube at 700 × *g* at room temperature for 2 min to remove the Equilibration Buffer.
53. Discard the flow-through from the 15 mL conical tube.
54. Place the white rubber cap on the end of the column to prevent loss of liquid.
55. Pipette up to 7 mL of the protein extraction solution into the column.
56. Incubate the column at room temperature for 30 min with gentle rocking.
57. Remove the white rubber cap and place the column back into the 15 mL conical tube. Loosen the cap on the column.
58. Allow the solution to drip through the column by gravity, emptying the 15 mL conical tube when necessary to prevent the flow-through from touching the tip of the column.
59. Centrifuge the column/conical tube at 700 × *g* at room temperature for 2 min to remove any residual protein extraction solution.
60. Discard the flow-through from the 15 mL conical tube, place column back into the conical tube.
61. Pipette 2 mL of Wash Buffer into the column.
62. Centrifuge the column/conical tube at 700 × *g* at room temperature for 2 min to remove the Wash Buffer.
63. Discard the flow-through from the 15 mL conical tube.
64. Repeat the above wash step 3 additional times.
65. After the final wash step, place the column into a clean 15 mL conical tube.
66. Add 1 mL of Elution Buffer to the column.

67. Centrifuge the column/conical tube at 700 × *g* at room temperature for 2 min to collect the Elution Buffer and eluted Protein A/G-MNase.
68. Add an additional 1 mL of Elution Buffer to the column.
69. Centrifuge the column/conical tube at 700 × *g* at room temperature for 2 min to collect the Elution Buffer and eluted Protein A/G-MNase. There is no need to collect the two elutions as separate fractions, they should be mixed.
70. Discard the column and store the eluted purified Protein A/G-MNase on ice.

Purification of MNase: Removal of imidazole from purified protein A/G-Mnase

Timing: 20 min

Zeba Spin Desalting Column used to remove excess imidazole from the purified Protein A/G-MNase.

71. Snap the tab off the bottom of a 10 mL Zeba Spin Desalting Column.
72. Place the column in a 50 mL conical tube and loosen the cap.
73. Centrifuge the column/conical tube at 1,000 × *g* at room temperature for 2 min to remove the storage buffer.
74. Discard the flow-through from the conical tube.
75. Pipette 5 mL of PBS into the column to equilibrate.
76. Centrifuge the column/conical tube at 1,000 × *g* at room temperature for 2 min to remove the PBS.
77. Discard the flow-through from the conical tube.
78. Repeat the PBS equilibration spins 3 additional times.
79. Place the column into a new 50 mL conical tube.
80. Slowly pipette the 2 mL purified Protein A/G-MNase sample directly onto the resin of the column.
81. Centrifuge the column/conical tube at 1,000 × *g* at room temperature for 2 min to elute the desalted purified Protein A/G-MNase from the column.
82. Pipette the desalted purified Protein A/G-MNase solution into a new 15 mL conical tube, store on ice.

Purification of MNase: Prepare protein standards for Bradford Assay

Timing: 10 min

BSA used to make a series of protein standards for use in a Bradford Assay.

83. Prepare 6 BSA protein standard solutions using a fresh ampule of 2.0 mg/mL BSA Protein and PBS as follows:
    a. Tube A: 100 µL PBS.
    b. Tube B: 100 µL BSA (no dilution).
    c. Tube C: 100 µL BSA + 100 µL PBS.
    d. Tube D: 100 µL of Tube C + 100 µL PBS.
    e. Tube E: 100 µL of Tube D + 100 µL PBS.
    f. Tube F: 100 µL of Tube E + 100 µL PBS.

Purification of MNase: Bradford Assay to determine protein concentration

Timing: 30 min

NanoDrop One Spectrophotometer used to determine the concentration of Protein A/G-MNase in sample.

84. Invert the bottle of Coomassie Plus (Bradford) Assay Reagent several time to mix.
85. Pipette 250 μL of Coomassie Plus (Bradford) Assay Reagent into each of six tubes labelled Tube A*-Tube F*.
86. Pipette 5 μL of each BSA protein standard prepared above into its corresponding tube of Coomassie Reagent.
87. Pulse vortex each tube several times to mix.
88. Incubate the tubes at room temperature for 10 min.
89. While the samples incubate, prepare the NanoDrop One Spectrophotometer for a Bradford Assay.
    a. From the **Home Screen**, choose the **Proteins** tab.
    b. Select **Protein Bradford**.
    c. Set the **Curve Type** to **2nd Order Polynomial**.
    d. Set replicates to **3**.
    e. Enter your standards into the chart.
        i. Tube A: 0 mg/mL.
        ii. Tube B: 2,000 mg/mL.
        iii. Tube C: 1,000 mg/mL.
        iv. Tube D: 500 mg/mL.
        v. Tube E: 250 mg/mL.
        vi. Tube F: 125 mg/mL.
90. Pipette 4 μL of Tube A (plain PBS) on the pedestal as a blank.
91. Measure 4 μL of each sample, as prompted by the spectrophotometer, to prepare the standard curve.
92. Prepare your experimental sample by pipetting 5 μL of the desalted purified Protein A/G-MNase sample into 250 μL of Coomassie Plus (Bradford) Assay Reagent.
93. Pulse vortex several times to mix.
94. Incubate the tube at room temperature for 10 min.
95. Pipette 4 μL of the sample solution onto the pedestal to determine the concentration of desalted purified Protein A/G-MNase.

Purification of MNase: Preparation of protein A/G-MNase solution for long-term storage

Timing: 10 min

Dilution of Protein A/G-MNase solution with glycerol.

96. Pipette 2 mL of sterile 80% glycerol to the 2 mL desalted purified Protein A/G-Mnase sample.
97. Gently pipette up and down several times to mix.
98. Pipette the protein solution into 200 μL aliquots.
99. Print labels for the tubes with the date of purification and concentration, remembering that the protein concentration is now half of what was determined using the Bradford Assay.
100. Freeze tubes at −20°C for long-term storage.

CUT&RUN: Prepare reagents

Timing: 15 min

Preparation of the all the solutions and reagents needed for the first part of the CUT&RUN protocol. Use the primary solutions prepared prior to starting the protocol.

101. Prepare Wash+ Buffer from pre-made Wash Buffer. Store on ice.
102. Prepare Dig-Wash+ Buffer using freshly made Wash+ Buffer. Store on ice.
103. Prepare Antibody Buffer using freshly made Dig-Wash+ Buffer. Store on ice.
104. Prepare the Concanavalin A beads for tissue binding. All steps are at room temperature.
    a. Thoroughly mix the Concanavalin A bead slurry by gently inverting the bottle several times.
    b. Pipette 15 μL of Concanavalin A bead slurry into each of two LoBind tubes (one tube for the experimental sample, one tube for a negative control).
    c. Resuspend the beads in 1 mL of Binding Buffer.
    d. Place the tubes in a magnetic rack and allow beads to migrate to magnet.
    e. Remove the supernatant.
    f. Repeat the Binding Buffer wash step once more, remove supernatant.
    g. Centrifuge the tubes briefly, return to magnetic rack. Remove all supernatant.
    h. Resuspend the beads in 1 mL of Wash+ Buffer.
    i. Store on ice.

CUT&RUN: Dissection of imaginal discs and binding of tissue to concanavalin A beads

Timing: 30 min

Preparation and collection of imaginal disc samples and binding to magnetic beads (Figure 1).

Note: Please see the troubleshooting section, problem 1 and problem 2 for help resolving certain issues related to this step of the protocol.

105. Place the two LoBind tubes containing the Concanavalin A beads in Wash+ Buffer on ice.
106. Depending upon the abundance of the chromatin mark being investigated, place 10–30 wandering 3rd instar larvae into a small petri dish containing sterile water and allow larvae to swim around for a few minutes to free the larval cuticle of excess food.

Note: Each larva contains two eye-antennal imaginal discs. If you are recovering other imaginal discs note that within a larva there are two labial discs, two clypeolabral discs, two wing discs, two haltere discs, six leg discs, and one genital disc.

107. Transfer each clean larva to a small pool (~1 mL) of Wash+ Buffer at the edge of a silicone-based dissection plate.

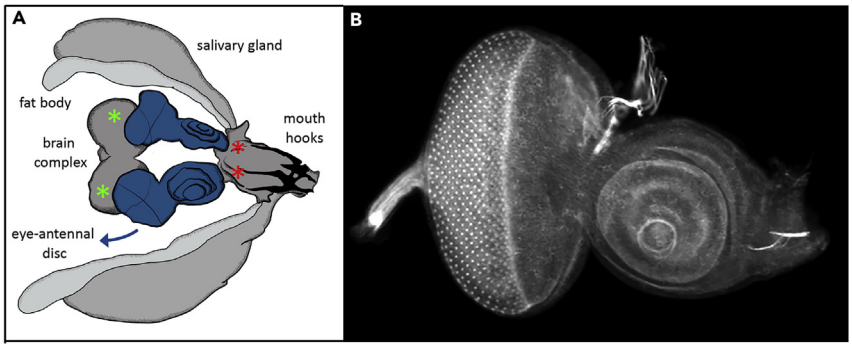

Figure 1. Dissection of imaginal discs and final product

(A) A diagram of the initial dissection product. It usually contains the mouth hooks, salivary glands, fat body, eye-antennal discs and brain complex. Green and red asterisks indicate areas that, when removed, will lead to the clean detachment of the eye-antennal disc.

(B) A light microscope image of a fully dissected eye-antennal disc. The tissue has been treated with phalloidin which attaches to F-actin. This reveals the overall morphology of the disc. Please see the written and video protocol in Spratford and Kumar, 2014 for a more detailed description of the dissection procedure.

108. Using forceps transfer a single larva to a large pool (~2 mL) of Wash+ Buffer in the middle of the same silicone-based dissection plate.
109. Using two pair of forceps, one in each hand, grasp and lightly hold the larva lengthwise with forceps.

Note: One pair of forceps should be used to gently but stably hold the larva approximately 1/3 of the body length from the mouth hooks. The other pair of forceps should be used to grab onto the mouth hooks.

110. Holding the mouth hooks steady, quickly but gently pull the body of the larva away from the mouth hooks. You should feel a slight release in tension as the larva tears apart.
111. Release the forceps holding the body of the larva and allow for the internal tissues remaining attached to the mouth hooks to spread out.
112. Immediately transfer the larval carcass (the portion without the mouth hooks, brain hemispheres, ventral ganglion, salivary glands and imaginal discs) out of the dissection bubble.
113. Holding the mouth hook complex firmly use a pair of forceps to remove the excess tissues including the salivary glands and overlying cuticle until a single complex of mouth hooks, brain hemispheres, ventral ganglion and imaginal discs remain.
114. Orient the remaining complex with the ventral side facing downwards.
115. Use one pair of forceps to again firmly hold the mouth hooks. With a second pair of forceps, remove the brain hemispheres by placing a single tong into the space between the brain and eye-antennal discs and rapidly pulling the brain away from the mouth hooks.

Note: For a visual demonstration of the dissection of imaginal discs please see the video protocol associated with Spratford and Kumar, 2014.

116. Isolate the remaining imaginal tissues by pinching off the discs as closely as possible to the connecting cuticle.
117. Once the imaginal discs are free from all other tissue transfer the newly dissected disc to the LoBind tube containing the Concanavalin A beads in Wash+ Buffer.
118. For abundant chromatin marks such as trimethylation of lysine 27 on histone H3 (H3K27me3) repeat above steps until 10 individual imaginal discs have been transferred to each of the two LoBind tubes. For less abundant chromatin marks such as trimethylation of lysine 4 on histone H3 (H3K4me3) repeat the above steps until 30 individual imaginal discs have been transferred to each of two LoBind tubes.
119. Incubate the tubes at room temperature with inversion for 10 min to allow the beads to bind to the tissue.

CUT&RUN: Primary antibody incubation

Timing: 10 min with 16 h incubation

Digitonin perforates tissue, allowing primary antibody to bind targets in the nucleus.

Note: Please see the troubleshooting section, problem 3 and problem 4 for help resolving certain issues related to this step of the protocol.

Note: The molecular portion this CUT&RUN protocol is adapted from the protocol developed by Kami Ahmad: https://www.protocols.io/view/cut-run-with-drosophila-tissues-umfeu3n and the EpiCypher CUT&RUN protocol: https://www.epicypher.com/resources/protocols/cutana-cut-and-run-protocol/.

120. Place the tubes in a magnetic rack and allow the bead-bound tissue to migrate to magnet for approximately 2 min. Collected tissue that has migrated to the magnet is clearly visible on the inside of the tube.
121. Remove all supernatant.
122. Briefly centrifuge the tubes in a benchtop mini-centrifuge to collect the tissue at the bottom of the tube, return tubes to the magnetic rack and allow tissue to migrate to magnet.
123. Remove all residual supernatant.
124. Add 100 μL of Antibody Buffer to the tissue pellet in each tube.
125. Add 2 μL of the primary antibody (100 μg/mL) (either Cell Signaling Technology Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit antibody or Sigma-Aldrich Trimethyl-Histone H3 (Lys4), Clone 15-10C-E4, Rabbit Monoclonal) to one tube. Final working concentration of the primary antibody is approximately 2 ng/μL.
126. Add 2 μL of the Cell Signaling Normal Rabbit IgG control antibody (1 mg/mL) to the second tube. Final working concentration of the control antibody is 20 ng/μL.
127. Mix the solution and resuspend the tissue pellets by gentle pipetting.
128. Incubate overnight for 16 h at 4°C with gentle shaking.

CUT&RUN: Prepare reagents

Timing: 1 h

Preparation of the all the solutions and equipment needed for the second part of the CUT&RUN protocol.

129. Prepare STOP-R Buffer from pre-made STOP Buffer.
130. Heat Eppendorf ThermoMixer F1.5 with ThermoTop Lid to 37°C.
131. Cool a refrigerated table-top centrifuge to 4°C.

CUT&RUN: Primary antibody wash

Timing: 10 min

Excess unbound antibody washed from the tissue.

132. Place the tubes in a magnetic rack and allow tissue to migrate to magnet.
133. Remove all supernatant.
134. Pipette 1 mL of Dig-Wash+ Buffer into each tube, invert several times to resuspend tissue.
135. Incubate at room temperature for 1 min.
136. Place the tubes in a magnetic rack and allow tissue to migrate to magnet.
137. Remove all supernatant.
138. Pipette 1 mL of Dig-Wash+ Buffer into each tube, invert several times to resuspend tissue.
139. Incubate at room temperature for 1 min.
140. Place the tubes in a magnetic rack and allow tissue to migrate to magnet.
141. Remove all supernatant.
142. Briefly centrifuge the tubes in a benchtop mini-centrifuge to collect the tissue at the bottom of the tubes, return tubes to the magnetic rack and allow tissue to migrate to magnet.
143. Remove all residual supernatant.

CUT&RUN: Nuclease tethering

Timing: 1 h, 5 min

Protein A/G-MNase binds to primary antibody.

Note: Please see the troubleshooting section, problem 5 for help resolving certain issues related to this step of the protocol.

Note: The concentration of the Protein A/G-MNase stock solution that was made in our lab is 800 ng/μL. We have found this starting concentration to be sufficient for providing robust and reliable chromatin cleavage following dilution.

Note: Our Protein A/G MNase stock was made in-lab by first transforming bacterial cells with the pAG/MNase plasmid (#123461) available from AddGene. This was followed by induction of protein production, protein extraction and column purification, and finally desalting of the purified protein. Concentration of the purified protein was determined by a Bradford assay. A full description for producing the Protein A/G-MNase from the AddGene plasmid can be found at the beginning of this protocol.

144. Prepare a fresh dilution of Protein A/G-MNase by pipetting 1 μL of the Protein A/G-MNase stock (800 ng/μL) into 1 mL of Dig-Wash+ Buffer. Mix by gentle vortexing.
145. Pipette 150 μL of the Protein A/G-Mnase dilution into each tube.
146. Resuspend the tissue pellets by gentle pipetting.
147. Incubate the tubes at 4°C for 1 h with gentle shaking.

CUT&RUN: Nuclease wash

Timing: 10 min

Excess unbound Protein A/G-Mnase washed from tissue.

148. Place the tubes in a magnetic rack and allow tissue to migrate to magnet.
149. Remove all supernatant.
150. Pipette 1 mL of Dig-Wash+ Buffer into each tube, invert several times to resuspend tissue.
151. Incubate at room temperature for 1 min.
152. Place the tubes in a magnetic rack and allow tissue to migrate to magnet.
153. Remove all supernatant.
154. Pipette 1 mL of Dig-Wash+ Buffer into each tube, invert several times to resuspend tissue.
155. Incubate at room temperature for 1 min.
156. Place the tubes in a magnetic rack and allow tissue to migrate to magnet.
157. Remove all supernatant.
158. Briefly centrifuge the tubes in a benchtop mini-centrifuge to collect the tissue at the bottom of the tubes, return tubes to the magnetic rack and allow tissue to migrate to magnet.
159. Remove all residual supernatant.

CUT&RUN: Chromatin cleavage and release

Timing: 2 h, 45 min

Protein A/G-Mnase nuclease activity activated by addition of calcium, cleaved chromatin released from tissue.

160. Resuspend the tissue pellets in 150 μL of Dig-Wash+ Buffer and place the tubes on ice for 5 min to chill.
161. Add 3 μL of 100 mM $CaCl_2$ (2 mM final concentration) to each tube and mix by gentle pipetting.
162. Immediately return the tubes to ice.

163. Incubated tubes on ice for 2 h.
164. Add 100 μL of STOP-R Buffer to each tube and mix by gentle pipetting.
165. Incubate the tubes in an Eppendorf ThermoMixer F1.5 at 37°C for 30 min with 500 rpm shaking to help cleaved DNA fragments exit the tissue.

CUT&RUN: Cleaved chromatin purification

Timing: 30 min

Cleaved chromatin fragments purified using the Qiagen MinElute PCR Purification Kit.

166. Using the refrigerated table-top centrifuge, centrifuge the tubes at 16,000 × *g* for 5 min at 4°C.
167. Place the tubes in a magnetic rack and allow the tissue to migrate to the magnet.
168. Transfer the supernatant from each tube, which now contains the cleaved chromatin, to two new LoBind tubes.
169. Pipette 1.25 mL of Buffer PB into each of the tubes.
170. Ensure that the color of the solution is yellow (correct pH).
171. For each sample, place a Qiagen MinElute spin column in a collection tube.
172. Transfer 800 μL of the chromatin solution to a Qiagen MinElute spin column.
173. Centrifuge at 18,000 × *g* for 1 min at room temperature.
174. Discard the flow-through from the collection tube, and reinsert the column.
175. Transfer the remainder of the DNA solution into the Qiagen MinElute spin column.
176. Centrifuge at 18,000 × *g* for 1 min at room temperature.
177. Discard the flow-through from the collection tube, and reinsert the column.
178. Pipette 750 μL of Buffer PE into the column to wash.
179. Centrifuge at 18,000 × *g* for 1 min at room temperature.
180. Discard the flow-through from the collection tube, and reinsert the column.
181. Centrifuge at 18,000 × *g* for 1 min at room temperature to dry the column.
182. Discard the collection tube and insert each of the columns into two new LoBind tubes.
183. Pipette 10 μL of Buffer EB directly onto the center of each MinElute column membrane.
184. Incubate the columns for 2 min at room temperature.
185. Centrifuge at 18,000 × *g* for 1 min at room temperature.
186. Discard the columns and store the eluted, purified chromatin fragments at −20°C.

CUT&RUN: Library production and NGS

Samples are submitted to the IU Center for Genomics and Bioinformatics, our in-house Genome Services Facility, for DNA library production and next-generation sequencing from the isolated chromatin fragments.

In our experience with over 100 samples, performing CUT&RUN using histone marks with broad domains yields purified chromatin fragments with concentrations of 200–300 pg/μL as measured by an Invitrogen Qubit 3 Fluorometer using the dsDNA high-sensitivity assay. For more narrow histone marks the concentration of purified chromatin fragments is not detectable by NanoDrop spectrophotometer, Qubit fluorometer, or Agilent TapeStation. However, even with undetectable concentrations, we have never encountered a sample that was unable to produce a quality DNA library.

DNA libraries are prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina from New England Biolabs. The DNA fragments obtained from the CUT&RUN protocol described here are used as-is for DNA library production. No further shearing or fragmentation is necessary.

Next-generation sequencing is carried out using Illumina NextSeq equipment at a read-depth of 15 M reads per sample.

EXPECTED OUTCOMES

If this protocol is successful, then replicate samples treated with histone specific antibodies will cluster together in a hierarchical clustered heatmap of Spearman's correlation. Similarly, replicate samples treated with the IgG control antibody will also cluster together. An example of a hierarchical clustered heatmap in which samples are treated with either the IgG or H3K27me3 antibody is provided in Figure 2. Similarly, if the protocol is successful, then clear chromatin peaks will be visible at the individual gene and whole genome-wide level. In Figure 3 we provide a view of H3K27me3 and H3K4me3 peaks along a region of the second chromosome as well as three individual genes. Genes such as *Act5C* that are constitutively expressed in the imaginal disc will have low levels of the repressive H3K27me3 chromatin mark and higher levels of the H3K4me3 activation mark. Genes such as *eyes absent* (*eya*) that are expressed in a spatially restricted pattern will have both activation and repressive chromatin marks. Lastly, a gene such as *knot* that is either expressed at very low levels or not at all within the disc will be highly decorated with H3K27me3 but will have only very low levels of the H3K4me3 mark.

QUANTIFICATION AND STATISTICAL ANALYSIS

CUT&RUN bioinformatic pipeline

Acquire necessary genomic sequences:

Download *Drosophila* (dm6) genome fasta (.fa) sequences from ensembl:

```
wget http://ftp.ensembl.org/pub/release-105/fasta/drosophila_melanogaster/dna/Drosophila_melanogaster.BDGP6.32.dna_sm.toplevel.fa.gz
```

Unzip genome sequence:

```
gunzip Drosophila_melanogaster.BDGP6.32.dna_sm.toplevel.fa.gz
```

Download *Drosophila* (dm6) annotated gene sequence (.gtf) from ensembl:

```
wget http://ftp.ensembl.org/pub/release-105/gtf/drosophila_melanogaster/Drosophila_melanogaster.BDGP6.32.105.gtf.gz
```

Unzip annotated gene sequence:

```
gunzip Drosophila_melanogaster.BDGP6.32.105.gtf.gz
```

Download *E. coli* genome fasta (.fa) sequences from ensembl:

```
wget http://ftp.ensemblgenomes.org/pub/bacteria/release-52/fasta/bacteria_12_collection/escherichia_coli_gca_001606525/dna/Escherichia_coli_gca_001606525.ASM160652v1.dna_sm.toplevel.fa.gz
```

Unzip genome sequence:

```
gunzip Escherichia_coli_gca_001606525.ASM160652v1.dna_sm.toplevel.fa.gz
```

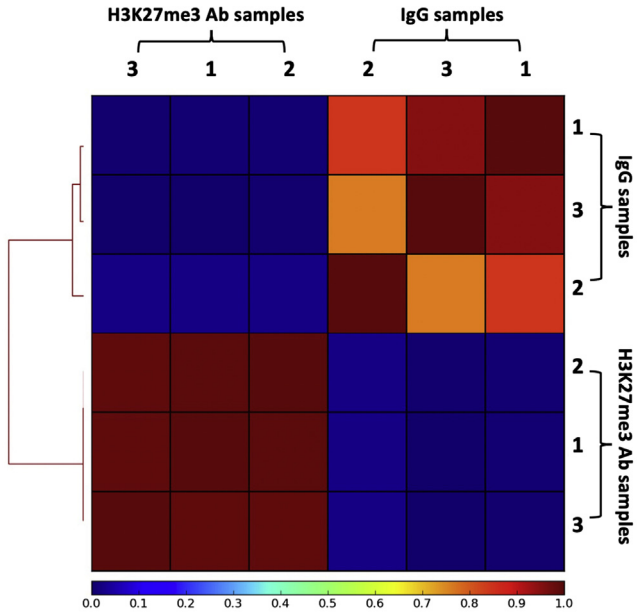

Figure 2. Read similarity with plotCorrelation

Hierarchically clustered heatmap of Spearman's correlation between samples with a histone specific antibody, H3K27me3, or the IgG control. Numbers below sample identifiers represent the sample number in each triplicate.

For this analysis you will need to install the following software (Note: numbers refer to version number of all packages):

`python/3.6.8, fastqc/0.11.9` (Andrews, 2010), `bowtie2/2.3.5.1` (Langmead and Salzberg, 2012), `samtools/2.2.0` (Li et al., 2009), `macs2/2.1.4` (Zhang et al., 2008), `deeptools/3.3.0` (Ramírez et al., 2016), `bedtools/2.29.2` (Quinlan and Hall, 2010), `sra-tools/2.10.3` (Leinonen et al., 2011).

`R packages – tidyverse/1.3.0` (Wickham et al., 2019), `getopt/1.20.3` (Davis and Day, 2019).

`Bioconductor packages – chipseeker/1.22.0` (Yu et al., 2015), `rtracklayer/1.46.0` (Lawrence et al., 2009), `genomicranges/1.38.0` (Lawrence et al., 2013), `genomicfeatures/1.38.0` (Lawrence et al., 2013).

Specific scripts found on rpolicastro, gzentner/ChIPseq, and zentnerlab/STRIPE-seq GitHub repositories:

```
git clone https://github.com/zentnerlab/ChIPseq.git
```

Bioinformatic analysis:

1. Run quality control on raw fastq files with `fastqc` (Andrews, 2010).
   a. Input: `fastq` reads.
   b. Output: `fastqc.html` reports.
   c. Expectation: Opening the html file will have eleven categories of quality control checks. Both reads of each sample should pass most, if not all, of these checks – particularly the basic statistics, per base sequence quality, per sequence quality scores, and per base sequence content.
2. Align fastq files to *Drosophila melanogaster* genome (dm6) index with `bowtie2` (Langmead and Salzberg, 2012).
   a. Input: raw fastq, Dm_genes.gtf, and Dm_genome.fa files.

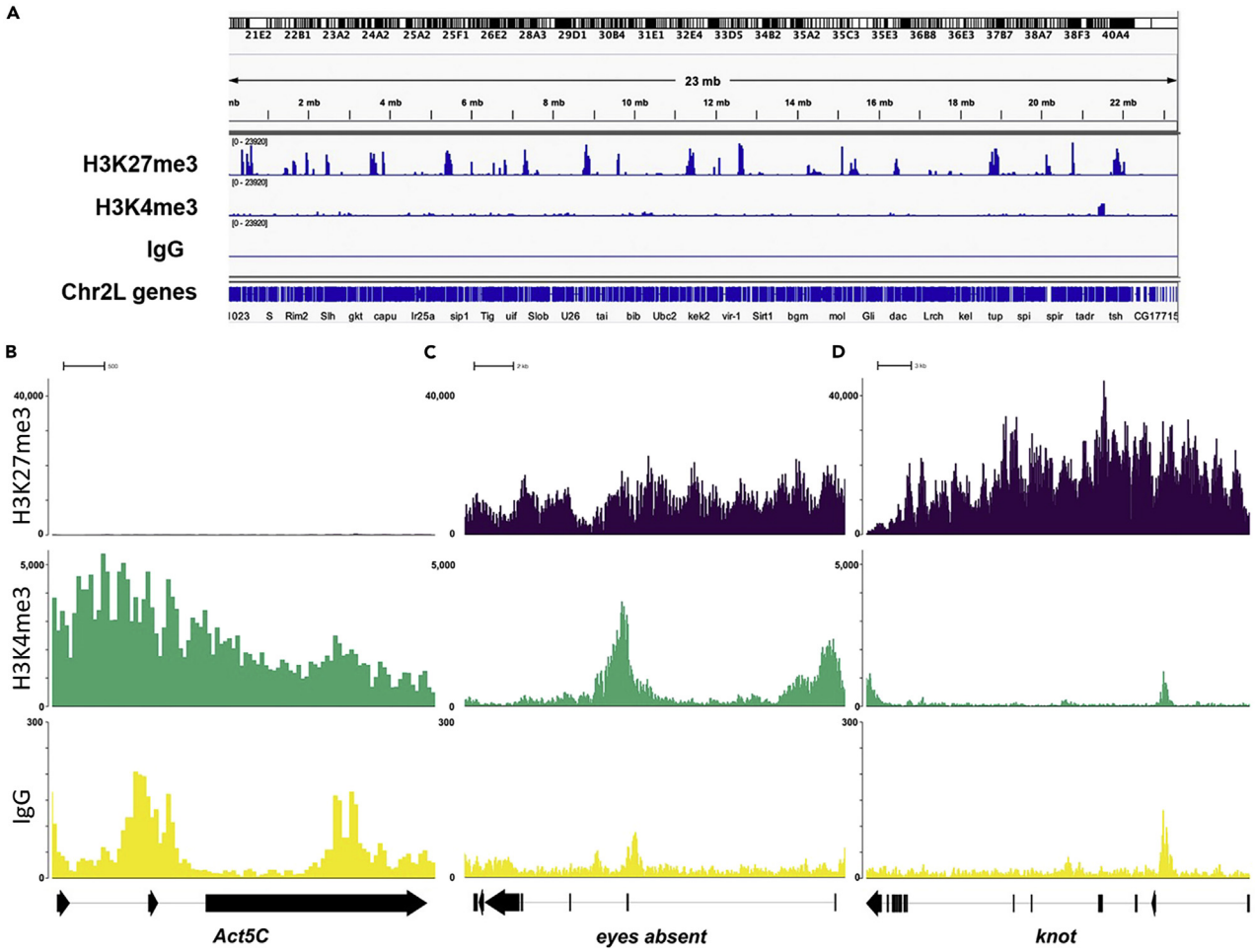

Figure 3. Visualizing chromatin peaks with IGV and Gvis

(A) Genome browser view of a normalized CUT&RUN wild-type eye-antennal disc signal spanning chromosome 2L.

(B–D) Gene locus tracks of (B) a constitutively active gene, *act5C*, (C) a developmentally regulated eye gene, *eya*, and (D) a gene important for wing disc patterning – and not expressed in the eye-antennal disc – *knot* (*kn*). For all tracks, (top) H3K27me3, (middle) H3K4me3, and (bottom) IgG control.

Note: See how to download genes.gtf and genome.fa files from ensembl to your terminal or supercomputer above.

b. Output: .sam, .bam, and .bai files.

3. Call Peaks with `MACS2` (Zhang et al., 2008) – use `narrowPeak/broadPeak` option depending on the histone mark of interest (i.e., H3K27me3 has a broad peak expression, while H3K4me3 is narrow).
   a. Input: `bam` file.
   b. Output: raw peak values (`peaks.xls` file).
   c. You can then use this as input for `ChIPSeeker` (Yu et al., 2015) or `NGSplot` (Shen et al., 2014) to generate transcription start site (TSS) or peak-centric heatmaps.
4. Combine raw bam files with `multiBamSummary` (Ramírez et al., 2016).
   a. Input: 3Ab experimental + 3IgG control `.bam` files.
   b. Output compressed `.npz` file (to be used in `plotCorrelation`).
      i. `multiBamSummary bins –bamfiles file1Ab.bam file2Ab.bam file3Ab.bam file1IgG.bam file2IgG.bam file3IgG.bam -o condition_name_results.npz`.
5. Analyze read similarity with `plotCorrelation` (Ramírez et al., 2016).

a. Input: `.npz` file.
   i. `plotCorrelation -in condition_name_results.npz --whatToPlot heatmap -CorMethod spearman -o condition_name_heatmap.png` *(or .pdf)* `-labels sample_1 sample_2 ...`
b. Output: heatmap.
c. Expectation: Quality reads should depict a cluster of the ab triplicate samples distinct from the cluster of IgG triplicate samples (Figure 1).

6. Calibrate samples to the *E. coli* genome (renders spike-in normalization unnecessary due to the *E. coli* DNA carried over during pAG-MNase purification:
   a. Align fastq reads to *E. coli* genome with `bowtie2` (Langmead and Salzberg, 2012).
      i. Input: raw `fastq` and `Ec_genome.fa` files.

      Note: *E. coli* genome.fa can be downloaded from ensembl (see above).

      ii. Output: `.sam, .bam, and .bai` files.
   b. Obtain the number of reads that align to the *E. coli* genome from the generated .bam file with `samtools` (Li et al., 2009): `samtools view -c -F 4 file.bam`.
   c. Use the generated aligned reads to calculate your scale factor.
      i. `[10,000/(#`*`E.coli`*` aligned reads)]`.
      For example, if it returned 624 counts, this would give you a scale factor of (10,000/624)=16.026.
   d. Normalize your original *Drosophila* aligned bam files and get tracks with `bamCoverage` (Ramírez et al., 2016).
      i. Input: *Drosophila* aligned `.bam` files.
      ii. Output: calibrated `bigwig` files.
      iii. `bamCoverage -bam Dm_file.bam -o file_name_normalized.bigwig -scaleFactor 16.026`.
7. To quickly visualize peaks, use Integrative Genomics Viewer (IGV) software (Figure 2A).
   a. Open raw/calibrated `bigwig` files.
   b. Select all 3 Abs experimental samples together → Group autoscale.
   c. Select all 3 IgG's control samples together → Group autoscale.
      i. This will generally show you overall if peaks are similar among the experimental and control replicates and if there are any outliers.
      ii. You can also choose genes to focus on to get a quick idea of what chromatin marks are there.
8. Or, use `Gvis` (Hahne and Ivanek, 2016) in RStudio to make the figures shown in this protocol (Figures 2B–2D).
   a. Template script on zentnerlab github (STRIPE-seq repository).

LIMITATIONS

There are a few instances in which the feasibility of this protocol could potentially be limited. We have suggested possible workarounds.

One limiting factor is that some epigenetic marks are less frequently placed along the genome. If you are attempting to determine the pattern of these chromatin marks we recommend increasing the number of imaginal discs that are used as starting material. For example, while ten discs were sufficient to determine the pattern of H3K27me3 marks, our protocol requires thirty discs be used to ascertain the pattern of H3K4me3.

The amount of input material is critical for the success of this protocol. A third larval instar eye-antennal disc contains approximately 44,000 cells. We can successfully detect the H3K27me3, H3K27ac, H3K4me3, and H2AK119Ub chromatin marks with 10–30 late-stage third instar imaginal discs. Younger eye-antennal discs from the first and second larval instar stages are significantly

smaller in size and contain considerably fewer number of cells. Similarly, the labial and clypeolabral imaginal discs are significantly smaller than eye-antennal discs even at the third larval instar stage. Please see Kumar, 2011 for a description of imaginal disc cell numbers. If you would like to use this protocol for smaller types of imaginal discs or for discs at early stages of development, you will need to adjust the number of discs that you dissect so that you will approach the ~450,000 or so cells required for this protocol.

Lastly, the physical structure and complexity of the tissue could limit the robustness of this protocol, which is designed to work on imaginal discs that are very thin and consisting of only two cell layers – a disc proper epithelium (DP) and a peripodial epithelium (PE). Please see Weasner and Kumar, 2022 for a textual and visual depiction of the structure of the eye-antennal imaginal disc. The overall structure and thickness of all other imaginal discs is like that of the eye-antennal disc. If you are using tissues that are considerably more dense than imaginal discs such as embryos and brains, this protocol may only result in the capturing of DNA fragments from the surface cells. We suggest manual disruption or mild sonication of the tissue prior to binding tissue to concanavalin A beads.

TROUBLESHOOTING

Problem 1

Third larval instar eye-antennal discs each contain approximately 44,000 cells. Other imaginal discs such as the haltere, labial and clypeolabrum discs contain far fewer number of cells. For example, the haltere disc contains between 7,500 and 10,000 cells. The other two discs are even smaller. Ten of these smaller discs might not provide enough material.

Potential solution

For smaller imaginal discs we recommend starting with higher numbers of imaginal discs – 50 discs might be a good starting point.

Problem 2

This protocol has been successfully used on eye-antennal discs that have been dissected at various stages during the third larval instar stage. Since the bulk of imaginal disc growth takes place during this last larval instar, younger discs (i.e., first and second larval instar), are significantly smaller in size and contain far fewer number of cells. As such, ten imaginal discs are unlikely to provide sufficient material for successful library preparation.

Potential solution

For younger imaginal discs we recommend starting with at least 50–100 imaginal discs and increasing to higher numbers of discs if necessary.

Problem 3

We have used this protocol successfully with antibodies that recognize abundant histone modifications such as trimethylation of lysine 27 on histone H3 (H3K27me3), acetylation of lysine 27 on histone H3 (H3K27ac), and ubiquitination of lysine 119 on histone H2A (H2AK119Ub). We obtained robust results with as few as ten eye-antennal imaginal discs. However, some chromatin marks such as trimethylation of lysine 3 on histone H3 (H3K4me3) or monomethylation of lysine 79 on histone H3 (H3K79me1) and are less frequently placed along the genome. As such, ten eye-antennal discs may not provide sufficient material for successful library preparation and sequencing. For example, we achieved successful detection of H3K4me3 by increasing the amount of starting material to thirty eye-antennal imaginal discs.

Potential solution

For low abundant marks we recommend starting with 30 discs and increasing to 50, 75, and 100 discs if necessary.

Problem 4

Several commercially available antibodies are available for each chromatin mark. Not all of them will provide high quality nucleosome peaks thus it is possible that DNA will not be recovered when some antibodies are used.

Potential solution

We recommend, when possible, using antibodies that have already been validated (in publications) for use with *Drosophila* imaginal discs. If that is not possible then we recommend evaluating different ChIP or CUT&RUN quality antibodies on a trial-and-error basis.

Problem 5

A lack of nucleosome peaks could also be caused by inefficient MNase digestion.

Potential solution

We recommend that you re-purify the Protein A/G-MNase enzyme as outlined in the accompanying MNase Purification Protocol.

If your laboratory is not equipped for protein purification, we recommend that you purchase the Protein A/G-MNase enzyme from a commercial source.

RESOURCE AVAILABILITY

Lead contact

Further information regarding dissections of imaginal discs should be directed to Bonnie M. Weasner (blooking@indiana.edu). Inquiries into the molecular treatment of the imaginal discs and DNA fragments should be directed to Brandon Weasner (bweasner@indiana.edu). Direct queries about the bioinformatic pipeline and statistical analysis to Haley E. Brown (hebrown@indiana.edu) and Bob Policastro (robert.policastro@egenesisbio.com). Questions about imaginal disc development should be directed to Justin P. Kumar (jkumar@indiana.edu).

Materials availability

The study did not generate new unique reagents.

Data and code availability

The genomic data sets supporting the current protocol have not been deposited in a public repository as it will be part of an upcoming study. However, they are available from the corresponding author on request. The code supporting the current protocol are available at Bob Policastro's GitHub (https://github.com/rpolicastro) and the Zentnerlab GitHub (https://github.com/zentnerlab).

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AUTHOR CONTRIBUTIONS

B.P.W. contributed to the design of the protocol, performed the experiments, and wrote and edited the manuscript. H.E.B. contributed to the design of the protocol, developed the bioinformatics pipeline, performed the experiments, analyzed the data, and wrote the manuscript. R.P. contributed bioinformatic scripts and edited the manuscript. B.M.W. contributed to the design of the protocol, performed the experiments, and wrote the manuscript. J.P.K. secured funding for the project and wrote and edited manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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