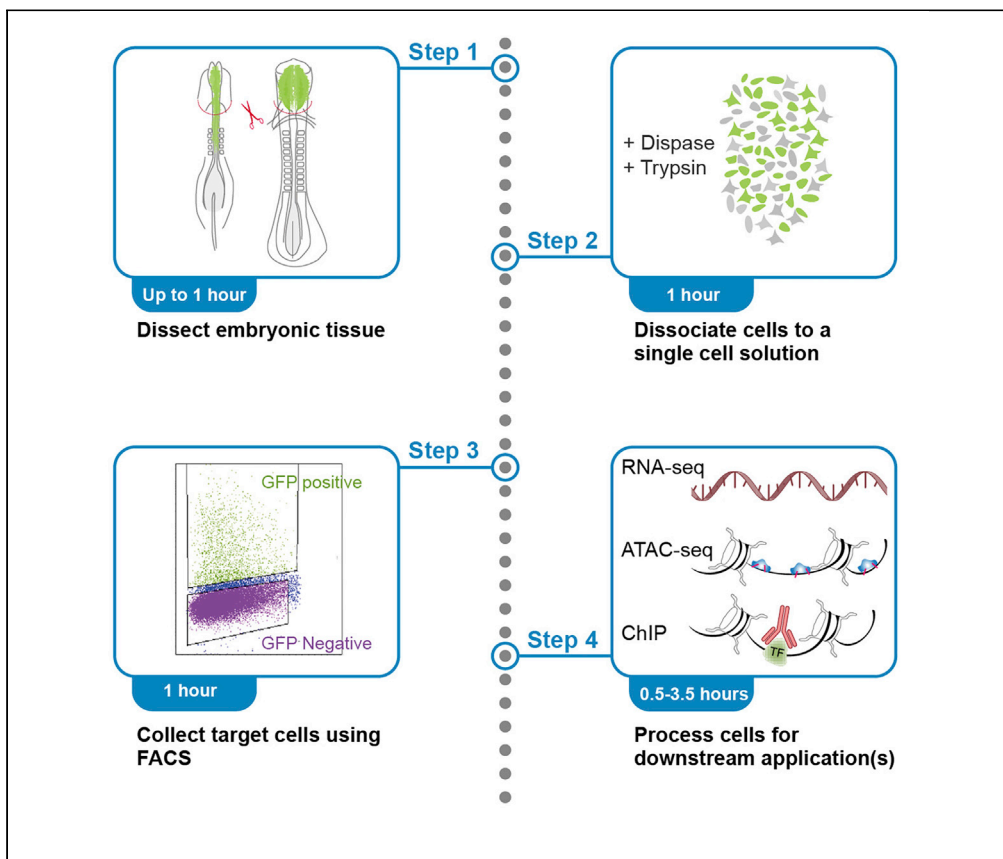


## Protocol

# Dissociation of chick embryonic tissue for FACS and preparation of isolated cells for genome-wide downstream assays



In order to process samples by fluorescence-activated cell sorting (FACS), it is essential to obtain a single-cell suspension of dissociated cells. Numerous protocols and commercial reagents are available; however, each requires optimization for specific tissue types. Here, we describe an optimized protocol for dissociating dissected chick embryos across a broad span of developmental stages. We also provide protocols for processing targeted cell populations isolated using FACS for ATAC-seq, RNA-seq, and chromatin immunoprecipitation.

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### HIGHLIGHTS

Combined enzymatic digestion and physical homogenization for tissue dissociation

Dissociate single-tissue dissections as well as large tissue collections

Preparation of FACS-isolated cells for numerous downstream experiments

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## Protocol

## Dissociation of chick embryonic tissue for FACS and preparation of isolated cells for genome-wide downstream assays

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## SUMMARY

In order to process samples by fluorescence-activated cell sorting (FACS), it is essential to obtain a single-cell suspension of dissociated cells. Numerous protocols and commercial reagents are available; however, each requires optimization for specific tissue types. Here, we describe an optimized protocol for dissociating dissected chick embryos across a broad span of developmental stages. We also provide protocols for processing targeted cell populations isolated using FACS for ATAC-seq, RNA-seq, and chromatin immunoprecipitation. For complete details on the use and execution of this protocol, please refer to Ling and Sauka-Spengler (2019) and Williams et al. (2019).

## BEFORE YOU BEGIN

## Prepare embryos with fluorescently labeled target cells

⌚ Timing: Previous day 2–6 h

1. This protocol was developed for isolation of embryonic neural crest (NC) cells. To fluorescently label this population we electroporated HH4 embryos (see 'Ex ovo electroporation of early chicken embryos' STAR protocol) with a NC-specific enhancer driving GFP (Simoes-Costa et al., 2012).

**Alternatives:** Target cells can also be identified by tissue-specific expression of fluorescent reporters or immunostaining with fluorescently conjugated antibodies against specific cell surface markers.

**Note:** In the context of the developing cranial neural crest cells, for example, we obtain 300–500 cells per embryo. Thus, cells obtained from multiple embryos are pooled to achieve the desired cell number (see downstream assays). We recommend performing a pilot experiment to establish how many cells/dissection can be obtained from the tissue of interest, then scale up accordingly. We routinely use this protocol for multiple (10–100) cranial dissections of HH8–10 chicken embryos (approx. 6 × 4 mm). This protocol is also suitable for single cranial dorsal neural tube dissections (approx. 3 × 2 mm). Older/larger embryonic dissections can also be processed in the same way (Ling and Sauka-Spengler, 2019).

2. Always prepare negative samples (non-fluorescent/unstained cells) and single-color control samples containing embryos expressing each fluorescent reporter separately in situations when



multiple fluorophores are used to isolate specific cell populations. These are necessary to establish gating parameters on the fluorescence-activated cell sorter (FACS machine) and adjust compensation if necessary.

### Pre-warm dispase solution and trypsin

⌚ Timing: 15 min

- Place aliquots (500  $\mu$ L of each per sample) of dispase and trypsin at 37°C for 15 min

### Prepare Hanks buffer

⌚ Timing: 15 min

- Prepare 12 mL of Hanks per sample to be processed

### Prepare Ringer's solution

⌚ Timing: 1 h

- Prepare stock solutions. Stock solutions can be stored at 18°C–22°C for 6 months.
- Prepare working solution. Working solution can be stored at 18°C–22°C for 6 months but once opened should be used on the same day or discarded to avoid cross contamination between experiments.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Biological samples</b>		
Chicken embryos, previously injected with construct of interests	N/A	N/A
<b>Chemicals, peptides, and recombinant proteins</b>		
Dispase®II (neutral protease, grade II)	Sigma	Cat #0494207800
Trypsin solution (0.05% trypsin/0.53 mM EDTA in HBSS, with sodium bicarbonate, without calcium and magnesium)	Thermo Fisher	Cat# MT25051C
10 $\times$ HBSS (Ca-, Mg-, phenol red-free)	Gibco	Cat# 14185-052
7-AAD	Thermo Fisher	Cat# 00-6993-50
Bovine serum albumin	Sigma	Cat# A3059
Hepes powder	Sigma	Cat# 90909C
<b>Critical commercial assays</b>		
Nextera DNA kit	Illumina	Cat #FC-121-1030
Nextera Index kit	Illumina	Cat #FC-131-1001
PCR Purification MinElute kit	QIAGEN	Cat #21894
NEBNext® Q5® HotStart HiFi 2 $\times$ PCR master mix	New England Biolabs	Cat #M0543
Ampure XP beads	Agencourt	Cat #A63880
Qubit	Life Technologies	Cat #Q32854
Kapa Library Quantification Kit	Kapa Biosystems	Cat #KK4903
RNAqueous micro total RNA extraction kit	Ambion	Cat #AM1931
<b>Experimental models: organisms/strains</b>		
Chicken embryos at desired stage, previously injected with construct of interests	N/A	N/A

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Other</i>		
1.7 mL Low-bind microcentrifuge tubes	SLS	Cat# DD039640T
ART® low-bind filter tips	VWR	Cat# 732-2219/732-2207/732-2355
Glass serological pipettes	Fisher	Cat# 7077-5N
High-performance 50 mL centrifuge tubes	VWR	Cat# 525-0447
40 µm Cell strainers	Corning	Cat# 352340
5 mL Round bottom polystyrene tubes	VWR	Cat# 60819-295
Tabletop mini centrifuge	N/A	N/A
Water bath	N/A	N/A
Low-speed centrifuge with swing-bucket rotors	N/A	N/A
Chilled centrifuge	N/A	N/A
BD FACS Aria Fusion cell sorter, or similar, with 100 µm nozzle	N/A	N/A
Thermocycler	N/A	N/A

## MATERIALS AND EQUIPMENT

### Hanks buffer

Amount for 50 mL	Reagent	Final concentration
5.0 mL	10× HBSS	1 ×
125 mg	BSA	0.25%
500 µL	1M Hepes pH 8	10 mM
44.5 mL	ddH <sub>2</sub> O	N/A

### Ringers solution

#### 40× stock solution 1

Amount (g) for 1 l	Reagent	Final concentration (M)
144.0	NaCl	2.5
14.8	KCl	0.2
9.0	CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.06
To a final volume of 1 l	ddH <sub>2</sub> O	N/A

#### 40× stock solution 2

Amount (g) for 1 l	Reagent	Final concentration (M)
144.0	NaCl	2.5
8.7	NaHPO <sub>4</sub> ·7H <sub>2</sub> O	0.03
0.8	KH <sub>2</sub> PO <sub>4</sub>	0.006
To a final volume of 1 l	ddH <sub>2</sub> O	N/A
Adjust to pH 7.4		

#### 1× working Ringers solution

Amount ( mL) for 4 l	Reagent	Final concentration (X)
100	40× solution 1	1
100	40× solution 2	1
To a final volume of 4 l	ddH <sub>2</sub> O	N/A

△ **CRITICAL:** Do not directly combine stock solutions as they will precipitate. Check pH and if necessary, readjust to pH 7.4, with HCl, bring final volume to 4 l with ddH<sub>2</sub>O. Filter, sterilize, and aliquot into 100–200 mL bottles, store at 18°C–22°C. Once opened aliquots should be used within a single experiment to avoid any cross-contamination.

## STEP-BY-STEP METHOD DETAILS

### Dissociate dissected embryonic tissue

⌚ **Timing:** 1 h to process four samples in parallel

These steps describe the enzymatic treatment and physical homogenization procedures to dissociate dissected tissue pieces into single-cell solution ready for FACS isolation of targeted cells.

1. Dissect tissue of interest (e.g., cranial region of HH8-10 embryos (Williams et al., 2019), in Ringers at 18°C–22°C, pipette into 1.7 mL low-bind microcentrifuge tube.
2. Briefly spin the tissue in a table top mini-centrifuge and remove as much Ringers as possible.
3. Add 500 µL of the pre-warmed Dispase solution and pipette up and down gently 10 times using 1000 µL ART low-bind tip.
4. Incubate for 15 min at 37°C, homogenizing every 5 min with a 1000 µL ART low-bind tip. Pipette 10–20 times against the wall of the tube to disrupt the tissue.

△ **CRITICAL:** Monitor dissociation by eye and adjust timing accordingly. Prolonged enzymatic digestion can affect cell integrity.

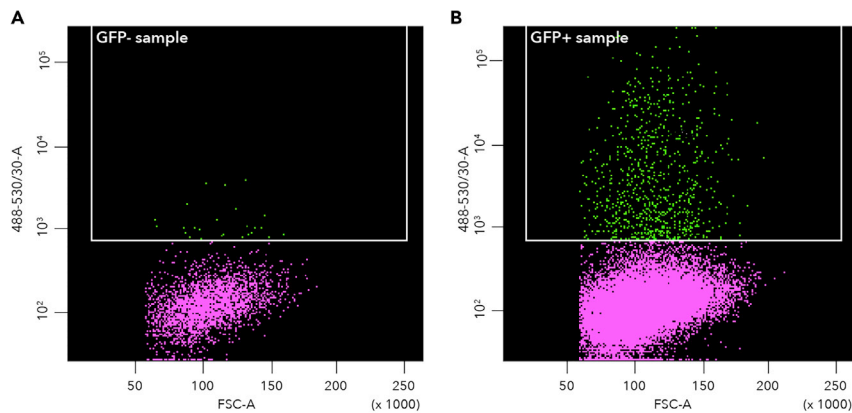
5. Add an equal volume (500 µL) of Trypsin solution, pipette up and down slowly to mix and incubate at 37°C for 3 min.
6. Stop the reaction by transferring the dissociated cells to 4 mL of Hank's solution in a 50 mL falcon tube.

△ **CRITICAL:** Ensure cell solution goes directly into Hanks solution; do not run down the side of the tube. The large volume of Hanks solution is necessary to allow thorough homogenization of cells, regardless of how little tissue is included. These washes are also important to remove phenol red (from Dispase and Trypsin solutions) which auto-fluoresces and may interfere with gating.

7. Rinse the microcentrifuge tube with 1 mL of Hank's solution and add to the cells in the 50 mL falcon tube.
8. Prepare a 5 mL glass serological pipette, coat the inside with Hank's solution by pipetting solution up and down several times. This minimizes loss of cells due to adhesion to the inside of pipette.
9. Homogenize the dissociated cells by pipetting up and down 10–15 times against the bottom of the tube, avoiding cavitation.
10. Centrifuge the dissociated cells at 500 g for 10 min at 18°C–22°C.
11. During this spin place a 40 µm cell strainer into a new 50 mL tube and apply 1 mL Hanks to moisten the strainer before cells are applied.

△ **CRITICAL:** Be at the centrifuge as soon as it stops and move 50 mL tubes very carefully to avoid disturbing the fragile pellet, ideally work at the centrifuge. Remove the supernatant slowly using a 10 mL pipette (do not pour off) ensuring no pellet is aspirated.

12. Re-suspend cells in 4 mL of Hanks solution using a pre-coated serological pipette as in step 8.



**Figure 1. Gating plots for collecting GFP-positive and -negative cells**

(A) Control sample, GFP-negative, derived from non-electroporated chick embryos.

(B) Experimental sample, GFP positive (green) and negative (magenta) cells isolated from chick embryos electroporated with neural crest enhancer, NC1, driving GFP.

13. Transfer the dissociated cells to the prepared 50 mL tube, by applying them to the moistened 40  $\mu$ m cell strainer. Rinse the 50 mL tube from which resuspended cells were removed with 1 mL of Hanks solution and add to cell strainer.
14. Centrifuge the 50 mL tube with cell strainer in place, at 750 g for 10 min at 18°C–22°C
15. Remove the supernatant, as in step 11, leaving approximately 300–500  $\mu$ L solution at the bottom of the tube to re-suspend the cells by gentle pipetting. Transfer to a 5 mL polystyrene round bottom tube used in the FACS sorter and keep on ice. Optional: Add 200  $\mu$ L Hanks to the 50 mL tube. After main sample has been FAC-sorted, this sample can be processed as well. Optional: The integrity and number of dissociated cells can be checked using a hemocytometer. Mix 10  $\mu$ L cells with 10  $\mu$ L of trypan blue solution and load onto a hemocytometer.
16. Add 1  $\mu$ L 7-AAD, or other compatible live-dead cell marker (7-AAD has broad excitation spectra, but emits at 647 nm and therefore cannot be used when detecting red fluorescent proteins).

**Alternatives:** For sorting in the red fluorescence range (mCherry, tdTomato), Hoechst 33258 solution can be used.

17. Prepare a few 1.7 mL low-bind tubes with  $\sim$ 20  $\mu$ L Hanks solution to collect cells into.

### Collect target cells by FACS

⌚ Timing: Approximately 1 h

Dissociated tissue is now ready for FACS, target cells are collected and processed according to the downstream application.

18. Proceed to FACS. The FACS machine should be set to maintain the sample at 4°C throughout sorting. If multiple samples are being processed keep these on ice.
19. Use control samples to establish gating parameters (Figure 1).
20. Collect negative (non-fluorescent) cells from the same sample as positive cells to use as controls in downstream assays.
21. Following FACS isolation of cells, proceed immediately to the downstream experiment or freeze using appropriate preservation method. Avoid leaving sorted cells on ice for excessive periods of time.

### Downstream applications: ATAC-seq

⌚ Timing: total 3–3.5 h (hands on = 2–2.5 h)

This section describes how to wash and lyse FACS sorted cells before performing Assay for Transposase Accessible Chromatin (ATAC), subsequent library preparation, clean-up steps and quality checks. This protocol has been modified from (Buenrostro et al., 2013). See this publication for full details of methodology.

22. Prepare ATAC lysis buffer on ice. Allow 50  $\mu$ L per sample.

Volume ( $\mu$ L)	Reagent	Final concentration
10.0	1M Tris-HCl pH 7.5	10 mM
2.0	5M NaCl	10 mM
3.0	1MgCl <sub>2</sub>	3 mM
10.0	10% NP40 (Igepal)	0.1%
975.0	DNase/RNase free ddH <sub>2</sub> O	

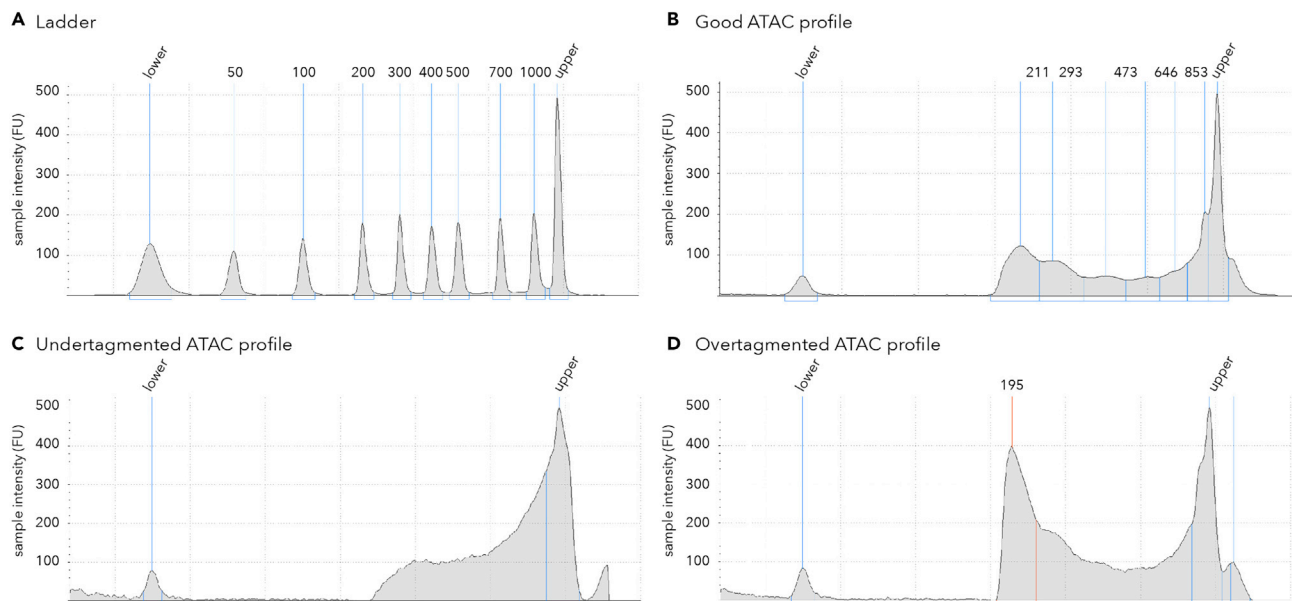
23. Place an aliquot of PBS on ice (50  $\mu$ L per sample) and allow to chill for approximately 15 min.
24. Centrifuge cells collected by FACS at 500 *g* for 5 min at 4°C.
25. Remove the supernatant by careful pipetting and wash the cell pellet with 50  $\mu$ L of ice-cold PBS and centrifuge as step in 24.
26. Remove the supernatant (PBS) and gently resuspend cells by pipetting 3–5 times in 50  $\mu$ L of ATAC lysis buffer, centrifuge at 500 *g* for 10 min at 4°C.
27. Prepare the transposition mix. The reaction detailed below is appropriate for 10,000 cells, scale up or down accordingly for different cell numbers.

**Note:** For less than 10,000 cells keep final volume at 10  $\mu$ L but adjust the amount of enzyme to avoid over-tagmentation (Figure 1D). The minimum number of cells we have successfully performed ATAC-seq with is ~250.

Volume ( $\mu$ L)	Reagent
5.0	2 $\times$ Tagment DNA Buffer, Illumina Nextera DNA kit Cat #FC-121-1030
0.5	<sup>a</sup> Tagment DNA enzyme, Illumina Nextera DNA kit Cat #FC-121-1030
4.5	RNase/DNase free ddH <sub>2</sub> O

<sup>a</sup>We have found new aliquots of enzyme can be “hyper active” and tend to over-tagment (Figure 2D). Optimization may be required. Enzyme activity may reduce over time and re-optimization will be required.

28. Remove lysis buffer and resuspend the cells in transposition mix.
29. Incubate the cells at 37°C for 30 min to tagment the DNA.
30. If working with cell numbers >2000, proceed directly to step 31. If working with cell numbers <2000 cells, it is recommended to add 1.1  $\mu$ L 0.5 mM EDTA to the reaction volume at the end of step 29 and incubate the tube for further 30 min at 50°C to stop the transposition reaction. This will promptly remove the transposase enzyme from the chromatin and prevent over-tagmentation of the chromatin. After 30 min at 50°C add 1.1  $\mu$ L of 0.5 mM MgCl<sub>2</sub> to the reaction, as EDTA chelates magnesium, which is essential for DNA polymerase activity. Proceed directly to step 32 as purification of low input samples can result in significant loss of material.
31. Immediately purify the reaction using Qiagen PCR purification MinElute kit. Elute transposed DNA in 10  $\mu$ L of elution buffer.



**Figure 2. Examples of ATAC profiles from Agilent TapeStation**

(A) TapeStation HS D1000 ladder.

(B) Appropriate tagmentation yields multiple peaks of accessible chromatin at approximately 150 bp intervals.

(C) Under-tagmentation results in predominantly large (>1 kb) fragments of chromatin.

(D) Conversely, over-tagmentation results in predominantly small (~200 bp) pieces of chromatin.

32. Setup the PCR amplification reaction as listed below.

Volume (μL)	Reagent
10.0	Transposed DNA
10.0	RNase/DNase free ddH <sub>2</sub> O
2.5	25 μM Universal PCR primer (Illumina Nextera Index kit #FC-131-1001)
2.5	25 μM Indexed <sup>a</sup> PCR primer (Illumina Nextera Index kit #FC-131-1001)
25.0	NEBNext <sup>®</sup> Q5 <sup>®</sup> HotStart HiFi 2x PCR master mix (#M0543)

<sup>a</sup>Use different indices for samples that will be pooled together for sequencing.

33. Amplify the transposed DNA fragments using the following cycling protocol.

Temperature (°C)	Time (S)	# cycles
98	30	N/A
98	10	11 <sup>a</sup>
65	15	
65	5	N/A
4	hold	N/A

<sup>a</sup>More cycles can be added to unpurified reaction if insufficient material is obtained from 11 cycles.

34. Assess tagmentation of samples on Agilent TapeStation or Bioanalyzer (Figure 2). Profiles should contain multiple peaks with maxima distributed roughly 150 bp apart (Figure 2B). The presence of majority of material in single peak, with sizes centered around the maximum of



~200 bp indicates over-tagmentation (Figure 2D) of the sample, whereas presence of majority of tagged material at ~1 kb mark indicates under-tagmentation (Figure 2C). In such cases timing of transposition reaction and concentration of Tn5 enzyme should be optimized. It is not uncommon to detect a large peak at ~40 bp pre-bead clean-up, as this represents the excess/abundance of primer dimers.

35. If samples cannot be detected by Tapestation or Bioanalyzer additional PCR cycles can be performed. During this assessment, the PCR reaction should be kept on ice.
36. Purify samples using Agencourt Ampure XP beads (A63880) to remove excess primers/primer dimers. We followed the manufacturers protocol which we describe below.
  - a. Vortex beads thoroughly for ~1 min to resuspend them.
  - b. Add 20  $\mu$ L of beads to the sample, mix thoroughly by pipetting up and down the solution 10 times using ART low-binding tips.
  - c. Incubate the sample with beads at 18°C–22°C for 5 min.
  - d. Pulse spin to collect the solution off the tube walls and place the tube in magnetic stand. Leave for 5 min for magnetic beads to separate out and for the solution to become clear.
  - e. While keeping the tube in the magnetic stand, remove and discard the supernatant. Proceed carefully not to remove/disturb the beads; if this happens, allow for beads to separate again.
  - f. Add 200  $\mu$ L freshly prepared 80% ethanol to the tube still in the magnetic stand to wash the pellet, leave for 30 s and remove. Repeat the wash step.
  - g. Air dry beads in the magnetic stand with lids open for ~5–10 min, until the pelleted beads change from shiny/glossy in appearance to a dull matt finish. Cracks should not be seen in the pelleted beads.
 

**△ CRITICAL: Over-drying causes problems in resuspending and subsequent loss of material.**
  - h. Remove tubes from the magnetic stand and elute DNA with 20  $\mu$ L of 0.1 $\times$  TE, resuspend by pipetting up and down at least 10 times. Keep at 18°C–22°C for 5 min.
  - i. Pulse spin, place in magnetic stand for 5 min.
  - j. Carefully remove ~18  $\mu$ L of supernatant containing DNA and transfer to a new tube.
37. Repeat Tapestation/Bioanalyzer analysis.
38. Determine the concentration of the eluted sample using Qubit assay (Life Technologies #Q32854). Following the manufacturers protocol.
39. Use Kapa Library quantification kit (Kapa Biosystems #KK4903) to quantify transposed DNA before sequencing. When sequencing multiple samples in the same lane/run, samples should be pooled such that they have equal molar amounts (4 nM each) before the Kapa quantification reaction and the pooled sample concentration should be assessed and adjusted.
40. Determine pooled library concentration using Kapa standards and dilute accordingly to either 4 nM, 2 nM, 1 nM or 0.5 nM, depending on the determined library concentration for sequencing on the Illumina NextSeq 500/550 platform, using 75 cycle high-output kit (#20024906). Always dilute to highest concentration possible.

### Downstream applications: Preserve cells in lysis buffer for RNA extraction

⌚ Timing: Washing, flash-freezing cells = 30 min

Here we describe how to wash, lyse, and store cells collected in bulk by FACS ready for subsequent RNA extraction and ultimately library preparation for RNA-seq. For RNA-seq library preparation, numerous kits are available depending on the quantity of input material used. For low-input samples (100's - 1000's of cells) we have used SMART-seq® V4 Ultra® low-input RNA kit for sequencing (Takara Clontech #634889) following the manufacturers protocol. To prepare the RNA-seq libraries we used Nextera XT kit (Illumina #FC131-1096).

For single-cell RNA-seq using SmartSeq2 (Picelli et al., 2014), cells should be collected individually in 96-well or 384-well plates (VWR #47743-996) containing cell lysis buffer (0.2% vol/vol Triton-X-100, 2U/μL RNase inhibitor) and the Oligo-dT<sub>30</sub>-VN primer (5'-AAGCAGTGGTATCAACGCAGAG-TACT<sub>30</sub>VN-3). These can be stored at -80°C for up to 3 months before following the published protocol (Picelli et al., 2014) for generating scRNA-seq libraries.

For sc-RNA-seq using the 10× Genomics Chromium platform, cells should be collected in bulk in a minimal volume of Hanks (0.25% BSA). Each cell collected from the FACS machine through a 100 μm nozzle comes in ~0.02 μL droplet. Refer to the Chromium single-cell 3' reagent kits v3 user guide (CG00183 RevC) to determine volume of cells to load on to the Chromium chip.

For sc-ATAC using the 10× Genomics Chromium platform, cells should be collected in bulk into Hanks buffer. Refer to Nuclei isolation for single-cell ATAC sequencing (CG000169 RevD) for downstream processing.

41. Centrifuge FACS isolated cells at 2000 g for 5 min at 4°C.
42. Carefully remove buffer by slowly pipetting and wash pelleted cells with 50 μL chilled (4°C) PBS. (depending on the number of cells the pellet may not be visible). Centrifuge as in step 41.
43. Carefully remove PBS by slowly pipetting and resuspend cells on ice by gentle pipetting in 100 μL chilled lysis buffer from Ambion RNaqueous micro total RNA extraction kit (AM1931).
44. Leave on ice 15 min with intermittent vortexing.
45. Briefly centrifuge, snap freeze in liquid nitrogen (this is recommended even if proceeding directly to RNA extraction).

**△ CRITICAL:** Carefully place tube(s) vertically in liquid nitrogen using large metal forceps and appropriate PPE, such that lysis solution containing cells doesn't spread over walls of the tube and remains at the bottom.

46. Store at -80°C. For downstream RNA-seq, do not store samples at -80°C longer than 3 months.

### Downstream applications: Lyse and cross-link cells for ChIP

⌚ **Timing: 90 min**

Here we detail the initial steps of chromatin immunoprecipitation (ChIP), whereby cells are lysed and chromatin is cross-linked and snap-frozen ready for the subsequent ChIP procedure.

47. Prepare Nuclei Extraction Buffer (NEB). 1 mL per sample.

Volume (μL)	Reagent	Final concentration
100.0	10% NP40	0.5%
50.0	10% Triton X-100	0.25%
20.0	1M Tris-HCl pH 7.5	10 mM
60.0	25 μM 100 mM CaCl <sub>2</sub>	3 mM
333.3	1.5M Sucrose	0.25M
2.0	1M DTT	1 mM
2.0	200 mM PMSF	0.2 mM
285.7	7× Complete Protease inhibitor stock	1×
1147.0	DNase/RNase free ddH <sub>2</sub> O	N/A

48. Centrifuge FACS isolated cells at 2000 g for 5 min at 4°C.
49. Carefully remove supernatant by pipetting and wash pellet with 500 µL of chilled PBS, (depending on the number of cells the pellet may not be visible). Centrifuge as in step 48.
50. Carefully remove the PBS and gently resuspend cells in 1 mL of NEB.
51. Transfer cells to a chilled Dounce homogenizer and lyse them with 20 strokes of pestle B, on ice, taking care to minimize cavitation.
52. Transfer the homogenate to new 1.7 mL low-binding tube. Spin briefly and tap tube on bench to remove any bubbles.
53. Pipette 973 µL of homogenate into a new 1.7 mL low-binding tube and add 27 µL of 37% formaldehyde (to a final concentration of 1% formaldehyde).
54. Nutate the tube(s) at 18°C–22°C for precisely 10 min.
55. Add 143 µL of 1M glycine to quench the formaldehyde, nutate at 18°C–22°C for 5 min.
56. Pellet cells at 2000g for 4 min at 4°C.
57. Prepare ice cold PBS supplemented with protease inhibitors (PBS/PI) by dissolving 1 mini EDTA-free protease inhibitor tablet in 10 mL PBS and adding 10 µL 1M DTT and 10 µL 0.2M PMSF.
58. After the spin (step 56), remove supernatant and gently resuspend pellet in 1 mL of PBS/PI by pipetting with 1000 µL low-binding tip ~10 times. Centrifuge as step in 56 and repeat PBS/PI washes for a total of 3 washes. The pellet will become invisible as the fixative is rinsed out.
59. After the final spin, remove supernatant and snap freeze crosslinked cells in liquid nitrogen and store at –80°C.

**△ CRITICAL:** Carefully place tube(s) vertically in liquid nitrogen using large metal forceps and appropriate PPE, such that cells remain pelleted and don't spread over walls of the tube.

60. Cells can be stored at –80°C for several months before proceeding with the ChIP assay. We have generated a separate detailed protocol for ChIP-seq ([Lukoseviciute, 2020](#)), but cells prepared following these instructions can be used in numerous protocols, including commercial kit based assays.

## EXPECTED OUTCOMES

Using 7-AAD or other live/dead stains allows to reproducibly recover live cells (~95% live cell purity). From dissected cranial regions of chick embryos at 5–7ss we typically obtain ~300 GFP+ NC cells/dissection, at 8–10ss we get ~500 NC cells. This will vary vastly depending on cell-specific markers.

## LIMITATIONS

As with any tissue dissociation protocol cells can easily be lost or die throughout the procedure. Here we have taken several steps to reduce those undesirable effects, however, it is advisable to perform a test run to determine the number of cells obtained from your tissue of interest before planning the downstream experiment.

This protocol has been optimized for chick embryonic cells; however, we have also developed variations of this protocol to dissociate tissue from zebrafish ([Lukoseviciute et al., 2018](#)) and lamprey ([Hockman et al., 2019](#)) embryos.

## TROUBLESHOOTING

### Problem 1

Tissue doesn't dissociate in Dispase/Trypsin

### Potential solution

Increase incubation time and homogenization in Dispase/Trypsin solution. Carefully monitor dissociation by eye until all aggregates disappear. Alternatively, collagenase can be used. Dissolve collagenase (Sigma, C8176) in trypsin solution (0.05%) to a final concentration of 20 mg/mL. We found

this step necessary for dissociation of zebrafish and lamprey embryonic tissues. Conversely other combinations of cell dissociation enzyme can be used, such as Accumax cell detachment solution (Sigma, Cat. # SCR006) or Liberase (Roche Cat # 05401127001), but conditions for use need to be optimized.

### **Problem 2**

Cells clogging sorter

### **Potential solution**

This can happen if the extent of tissue dissociation is not carefully assessed and can be remedied by passing the cell suspension through a 70  $\mu\text{m}$  filter-capped 5 mL round bottom FACS tube (VWR, 21008-948).

### **Problem 3**

No cells detected by sorter

### **Potential solution**

Ensure samples are fluorescently labeled prior to running the FACS. Pellet could have been lost during dissociation. Take care to remove supernatants slowly and visually inspect pellet during the process. Supernatant can be re-collected and centrifuged again if necessary.

### **Problem 4**

Low cell viability

### **Potential solution**

This indicates cells have been stressed throughout the protocol. Be sure to work swiftly, pipette gently, and to not over-treat the sample with dispase/trypsin solution.

### **Problem 5**

ATAC samples are over or under-tagmented

### **Potential solution**

As shown in [Figure 1](#), tagmentation conditions may require some optimization. Adjust concentration of enzyme in tagmentation reaction. This may be more easily achieved by adjusting final volume, e.g., if 10,000 cells are over-tagmented in a 10  $\mu\text{L}$  reaction containing 0.5  $\mu\text{L}$  enzyme, the final volume of the reaction can be increased to 20  $\mu\text{L}$ , for example, and/or reducing enzyme volume used reduced to 0.25  $\mu\text{L}$ . Conversely if samples are under-tagmented more enzyme should be added or the final reaction volume reduced. The timing of tagmentation can also be adjusted accordingly.

## **RESOURCE AVAILABILITY**

### **Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tatjana Sauka-Spengler ([tatjana.sauka-spengler@imm.ox.ac.uk](mailto:tatjana.sauka-spengler@imm.ox.ac.uk)).

### **Materials availability**

This study did not generate new unique reagents.

### **Data and code availability**

This study did not generate/analyze datasets/code.

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## AUTHOR CONTRIBUTIONS

Methodology, R.M.W. and T.S.-S.; investigation, R.M.W. and T.S.-S.; resources, T.S.-S.; writing – original draft, R.M.W. and T.S.-S.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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