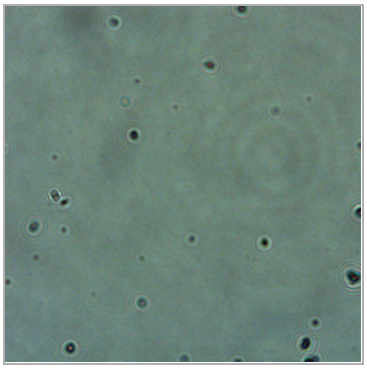

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A versatile nuclei extraction protocol for single cell multiome ATAC and gene expression in non model species

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DISCLAIMER

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ABSTRACT

Here we present a modified version of : dx.doi.org/10.17504/protocols.io.261genwm7g47/v2 that was used to successfully extract nuclei from an array of different tissue types for single cell sequencing and modify it with the purpose of extracting nuclei for single cell multiome ATAC and gene expression on the 10x chromium. The modifications for this protocol include: different concentration of RNase inhibitors, different quantities for nuclear isolation buffer, removal of unnecessary steps as well as QC specific for multiome analysis. If you are looking to use this protocol for bulk ATAC-seq use of protease inhibitor cocktail PIC is recommended instead of RNase inhibitor on the snRNA-seq version of this protocol (dx.doi.org/10.17504/protocols.io.261genwm7g47/v2) please get in touch with the authors if you are unsure on how to do this.

GUIDELINES

Before using this prep for library preparation do a trial run.
It is recommended to conduct a trial, especially on a new tissue type, to adjust various parameters without introducing RNase. This allows for the adjustment of parameters such as mincing times, filter size, and dilution into the final buffer, which ultimately leads to the production of good quality nuclei.

For multiome snRNA-seq and single cell ATAC-seq, it is advisable to perform a trial run where the quality of the nuclei is assessed by examining their integrity and the quality of their nuclear membrane (as shown in the picture in step 2.1).

MATERIALS

MATERIAL

Noyes Spring Scissors - Tungsten Carbide **Fine Science Tools Catalog #15514-12**

Tungsten Carbide Straight 11.5 cm Fine Scissors **Fine Science Tools Catalog #14558-11**

40 µm Falcon™ Cell Strainers **Fisher Scientific Catalog #08-771-2**

Corning™ Falcon™ Test Tube with 35µm Cell Strainer Snap Cap **Corning Catalog #352235**

pluriStrainer Mini 20 µm (Cell Strainer) **pluriSelect Catalog #43-10020-50**

X500 Eppendorf DNA LoBind Tubes, 1.5ml, PCR clean

Cryotube

6-well tissue culture plate (Stem Cell Technologies)

Falcon tubes 15 ml (Corning)

INCYTO C-Chip™ Disposable Hemacytometers **VWR International Catalog #82030-468**

SAMPLING AND STORAGE FOR NUCLEAR ISOLATION

Animals must be appropriately euthanized and immediately processed. Approximately ~ 60 mg of salmonid tissue is placed in one clearly labelled cryotube and immediately flash frozen in liquid nitrogen. **This step is critical**. The tissue must be preserved as fast as possible for optimal results. In the absence of liquid nitrogen, samples can be frozen in dry ice. Samples can be stored at -80 °C for up to a year prior to use. Older samples might still yield viable nuclei but this would need to be tested.

REAGENTS

All reagents should be chilled on ice prior to use.

2X stock of salt-Tris solution makes 10 mL:

Stocks:

NaCl: NaCl (5 M) RNase-free **Thermo Fisher Scientific Catalog #AM9759**

Tris-HCl pH 7.5:

UltraPure™ 1 M Tris-HCl Buffer, pH 7.5 **Thermo Fisher Catalog #15567027**

CaCl2:

Calcium chloride 1 M in aqueous solution **VWR International Catalog #97062-820**

MgCl2:

Magnesium chloride solution for molecular biology (1.00 M) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M1028**

Nuclease-free water:

Water for biotechnology nuclease-free sterile **VWR International Catalog #97062-794**

	A	B	C
	Stock solution (see above)	Volume	Final concentration
	NaCl	292 ul	146 mM
	Tris-HCL10	100 ul	10 mM
	CaCl2	10 ul	1 mM
	MgCl2	210 ul	21 mM
	Nuclease-free water	9388 ml	

The following buffers contain RNAase inhibitor

Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #3335399001**

- It is important to use the correct RNAse inhibitor as it can negatively affect library prep, check with the sequencing platform before using another type of RNAse.
- Do not add RNAse until right before nuclear extraction.
- RNAse inhibitor does not need to be used to test nuclear extractions, but it should added for sequencing runs.

1X ST buffer solution (**ST)** - 10 mL:

Dilute 2x ST in ultrapure nuclease-free water (1:1)

	A	B	C
	Stock Solution	Volume	Final concentration
	2X ST	3 ml	
	Ultrapure nuclease free water	3 ml	
	RNAse inhibitor	250 µl (240 U)	200 Uml

Make fresh and chill prior to use, add RNAnase inhibitor right before nuclear isolation. RNAase inhibitor amount can up upped if it's an RNAse Rich tissue, up to 500 U per ml instead, tissue spends very little time in this buffer and is chilled at all time, which is why the amount of RNAase inhibitor can be lower.

Working solution (**TST**) – 4 mL:

1% Tween-20:

Tween-20 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P-7949**

2% BSA:

Bovine Serum Albumin (20 mg/mL) Molecular Biology Grade **New England Biolabs Catalog #B9000S**

	A	B	C
	Stock solution	Volume	Final concentration
	2X ST buffer	2 ml	
	1% Tween-20	120 µl	
	2% BSA	20 µl	
	Nuclease-free water	1810 µl	
	RNAse inhibitor	50 µl	1000 Uml

Make fresh and chill prior to use, add RNAase inhibitor right before nuclear isolation .Dilute the Tween from 10% in stock solution with nfH2O before making the buffer. RNAnase inhibitor amount can be upped if it's an RNAase rich tissue up to 1000 U per ml instead, the nuclear isolation will happen in this buffer so its more critical in here.

Final dilution buffer will be stated on the protocol that the authors chose to use going forward, This protocol was developed using 10x multiome assay. RNAse inhibitor should be of concentration 1000 U/Ml, the RNAse inhibitor used should be protector RNase inhibitor (sigma-Aldrich)

Sampling and storage for nuclear isolation.
Animals must be appropriately euthanized and immediately processed. Approximately ~ 60 mg of tissue is placed in one clearly labelled cryotube and immediately flash frozen in liquid nitrogen. **This step is critical**. The tissue must be preserved as fast as possible for optimal results. In the absence of liquid nitrogen, samples can be frozen in dry ice. Samples can be stored at -80 °C for up to a year prior to use. Older samples might still yield viable nuclei but this would need to be tested.

All reagents should be chilled on ice prior to use.
Samples should be kept frozen on dry ice until immediately before nuclei isolation, and all sample-handling steps should be performed on ice.
The centrifuge should be pre chilled at 4 °C.

All reagents are given for 2 nuclear isolations.
Amounts of buffer especially those that contain RNase should be adjusted appropriately for each experiment prepared prior and RNase added immediately before use.

Nucleus isolation workflow for ST-based buffers

30m

1

Note

Samples should be kept frozen on dry ice until immediately before nuclei isolation, and all sample-handling steps should be performed On ice. The centrifuge should be pre-chilled at 4 °C.

On ice, place a piece of frozen tissue into one well of a 6-well tissue culture plate with 1 mL TST.

Note

If the sample is stuck to the cryotube, remove using tweezers, preferably while still in dry ice, and place immediately into the culture plate with TST. If the sample needs processing for examples cutting this is best done on dry ice. This is avoided by processing the sample prior to flash freezing.

2 On ice, mince tissue initially using Tungsten Carbide scissors for 00:00:30 and then with Noyes Spring Scissors Noyes Spring Scissors - Tungsten Carbide **Fine Science Tools Catalog #15514-12** for a total of 00:10:00. 10m

Note

This step is only necessary for fin, skin or similar hard tissues, for softer tissues just use spring scissors for 00:10:00.

2.1 00:05:00 into the mincing gently pipette up and down with a p1000 pipette using a low retention filtered tip. The time in the dissociation buffer is critical. See image for how to assess the timing is correct by looking at your nuclei. 5m

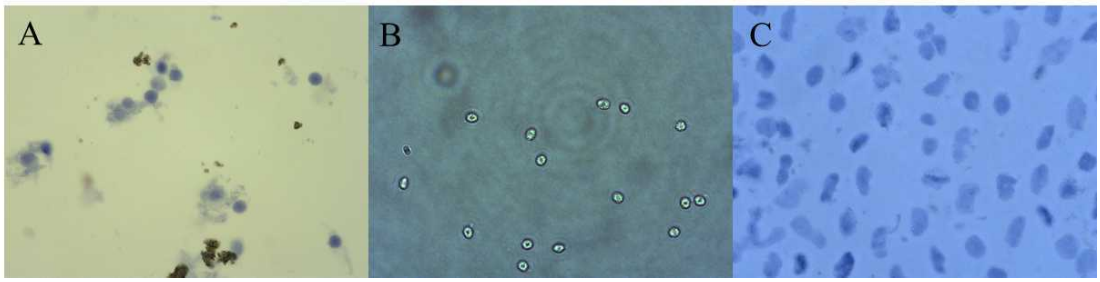

Image from different dissociation trials in Atlantic salmon tissues x40 magnification stained with trypan blue. **A**. Head kidney nuclei not had sufficient time in dissociation buffer, will clog microfluidic device. **B**. Blood nuclei perfectly dissociated minimal clumping ideal for sequencing. **C**. Liver nuclei to long in dissociation buffer, nuclear membrane started to degrade. Can still be sequenced but not ideal. Note when staining nuclei with trypan blue asses nuclear quality as soon as possible as the nuclei will quickly degrade when not on ice.

3 Pass lysate through a 40 µm cell strainer .

3.1 Add a further 1 mL of TST to the cell strainer immediately.

3.2 Add 3 mL of freshly prepared ST buffer to the lysate.

3.3 Add the 5 mL of lysate to a marked 15 ml falcon tube (Corning) on ice.

4 Centrifuge at 500 x g, 4°C, 00:05:00 in a swinging bucket centrifuge. 5m

5 Discard liquid, carefully remove excess liquid with a p200 pipette, careful to not disturve the pellet. Resuspend the pellet gently using a p1000 pipette in diluted nuclei buffer in aiming for target recover of 6.000 nuclei. The concentration of RNAse inhibitor should be 1U/ul.

Note

Resuspension volume depends on the size of the pellet, usually within the range of 100 µL - 1000 µL (1 mL if there are many nuclei). For skin and fin, 400 µL is recommended.

7/v2

6 Count the nuclei using a C-chip disposable haemocytometer.

Note

In this step, it is also possible to visualise the nuclei and ascertain the level of debris present as well as the integrity of the nuclear membrane.
Alternativly a non-disposable haemocytometer can be used.

7 The nuclei are also counted using a Bio-Rad TC20 to confirm results from the disposable haemocytometer and to count the proportion of viable cells.

Note

Nuclei are identified as “dead”, therefore a good nuclei isolation will have a small percentage of live cells. 1-4% of live cells is ideal. High proportions of live cells indicates incomplete nuclear isolation and could be an indication of high amounts of debris or insufficient lysis time.