A versatile nuclei extraction protocol for single nucleus sequencing in fish species – optimization in various Atlantic salmon tissues. V.1

Version 1

Jul 25, 2022

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DISCLAIMER

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ABSTRACT

Single cell RNA sequencing has rapidly become a standard tool for profiling transcriptomic diversity across thousands of cells (Linnarsson and Teichmann, 2016), and is now being applied to a large diversity of species and tissues. The main limitation of this technology is that it requires the isolation of live cells from fresh tissue, severely restricting its applicability. As a result, single nuclei RNA sequencing (snRNA-seq), which consists of sequencing the RNA of only the nuclei of cells rather than of the whole cell, has been commonly adopted since it allows samples to be stored for several months prior to processing while yielding comparable results to whole cell sequencing (Kulkarni, et al., 2019; Slyper et al. 2021). A critical challenge for snRNA-seq is the successful extraction of high quality nuclei. This has spurred the recent publication of a number of dissociation protocols for nuclei extraction (Drokhlyansky et al. 2020; Eraslan et al. 2021; Melms et al 2021), however, these have largely been optimized for model species such as humans, and more and more single nuclei is being adopted in non-model species.

Here we present a robust protocol that enables the extraction of nuclei from frozen tissue adapted from those shown to work in different tissue types, such as human skin (Drokhlyansky et al. 2020; Eraslan et al. 2021; Melms et al 2021). Our protocol has been used to successfully extract nuclei from an array of different Atlantic salmon (Salmo salar) tissues including skin, fin, spleen, head kidney, liver and gill as well as in other species such as sole (Solea solea) and rabbit (Oryctolagus cuniculus) nasal tissue and nurse shark (Ginglymostoma cirratumspleen). We present the protocol as applied to fin and skin as these are particularly challenging tissues to work with given their toughness and the presence of hard tissue (e.g., scales and bones), connective tissue and fat deposits. We include notes throughout the protocol so that the reader can optimise it for a variety of tissue types. While the protocol has been optimised to work with the Chromium 10x platform, the most commonly used high throughput microfluidic device, but can be used successfully for the extraction of nuclei for other platforms and applications. The aim of this protocol is to capture 7,000 nuclei per single-nuclei RNA sequencing library using the Chromium Single Cell 3' Reagent Kits v2 or v3 (10X Genomics). Given its utility for isolating nuclei from difficult to dissociate tissue types, we anticipate that this protocol will be broadly applicable for snRNA-seq of non-model organisms and unconventional tissue types.

ATTACHMENTS

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GUIDELINES

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MATERIALS TEXT

MATERIAL

Noyes Spring Scissors - Tungsten Carbide **Fine Science Tools Catalog #15514-12** Step 2

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

Falcon™ Cell Strainers **Fisher** **40 µm** **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 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 (5 M) RNase-free **Thermo Fisher** NaCl: **Scientific Catalog #AM9759**

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

Calcium chloride 1 M in aqueous CaCl2: solution **Vwr Catalog #97062-820**

MgCl2:

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

Water for biotechnology nuclease-free Nuclease-free water: sterile **Vwr Catalog #97062-794**

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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 **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	5 ml	
Ultrapure nuclease free water	5 ml	
RNAse inhibitor	5.2 µl (160 U)	40 Uml

Working solution (**TST**) – **4 mL** :

Tween-20 **Sigma-**

1% Tween-20: **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	1 ml	
1% Tween-20	60 µl	
2% BSA	10 µl	
Nuclease-free water	930 µl	
RNAse inhibitor	2.08 µl (160 U)	40 Uml

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PBS+0.02 BSA (**PBS+BSA**) – **1 mL** :

A	B	C
Stock solution	**Volume**	**Final concentration**
Ultra-pure molecular grade PBS	990 µl	
2% BSA **	10 µl	
RNAse inhibitor	0.6 µl	40U ml

** can top this up this to 2% BSA if the cells are clumping or look degraded

DISCLAIMER:

BEFORE STARTING

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.

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** .

Nucleus isolation workflow for ST-based buffers 30m

1

Samples should be kept frozen on dry ice until immediately before nuclei isolation, and all sample-

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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.

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.

2 10m 30s

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** .

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 5m

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.

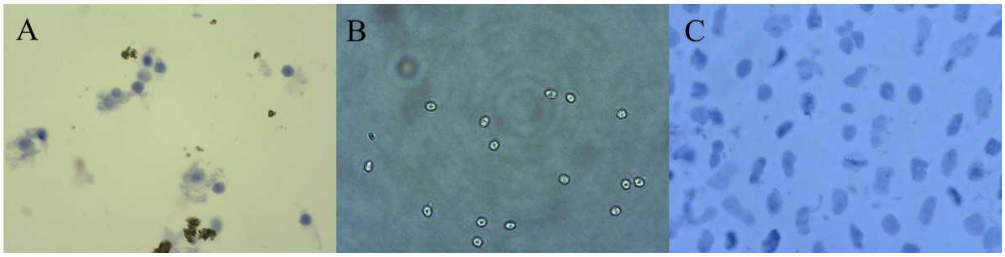

Image from different dissociation trials in Atlantic salmon tissues x40 magnification stained with trypan blue. **A**. Head kidney nuclei not had sufficient time in dissociation buffer, will clog microfluidic device. **B**. Blood nuclei perfectly dissociated minimal clumping ideal for sequencing. **C**. 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 .

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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 5m

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

5

Resuspend the pellet gently using a p1000 pipette in PBS-BSA.

Resuspension volume depends on the size of the pellet, usually within the range of **100 µL** - **500 µL** (**1 mL** if there are many nuclei). For skin and fin, **200 µL** is recommended.

6 Filter the nucleus solution a second time.

The size of the filter is tissue dependant, e.g. for tissues such as liver and head kidney a **40 µm** Falcon™ cell strainer will suffice, whereas for gill, a **30 µm** filter would be better giving the higher amount of tough debris that could clog the microfluidic device. In addition, for harder tissues that produce a lot of debris such as fin and skin (this is due to the presence of fat layers and scales in skin and the presence of bones in the fin) then **20 µm** is recommended. The lysate may not pass through at once, pipetting very gently up and down with a wide bore pipette can help it through.

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

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.

8 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.

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, but below 12% is acceptable. High proportions of live cells indicates incomplete nuclear isolation.

9 Load the nucleus suspension into a Chromium Chip and into the Chromium Controller, aiming to recover 7,000 nuclei as per 10x recommendations with a concentration of between 700 to 1200 nuclei per µl.

In the case of some tissues such as fin, readjust the target recovery to 5000 especially with juvenile fish for which nuclei yields are sometimes low.

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