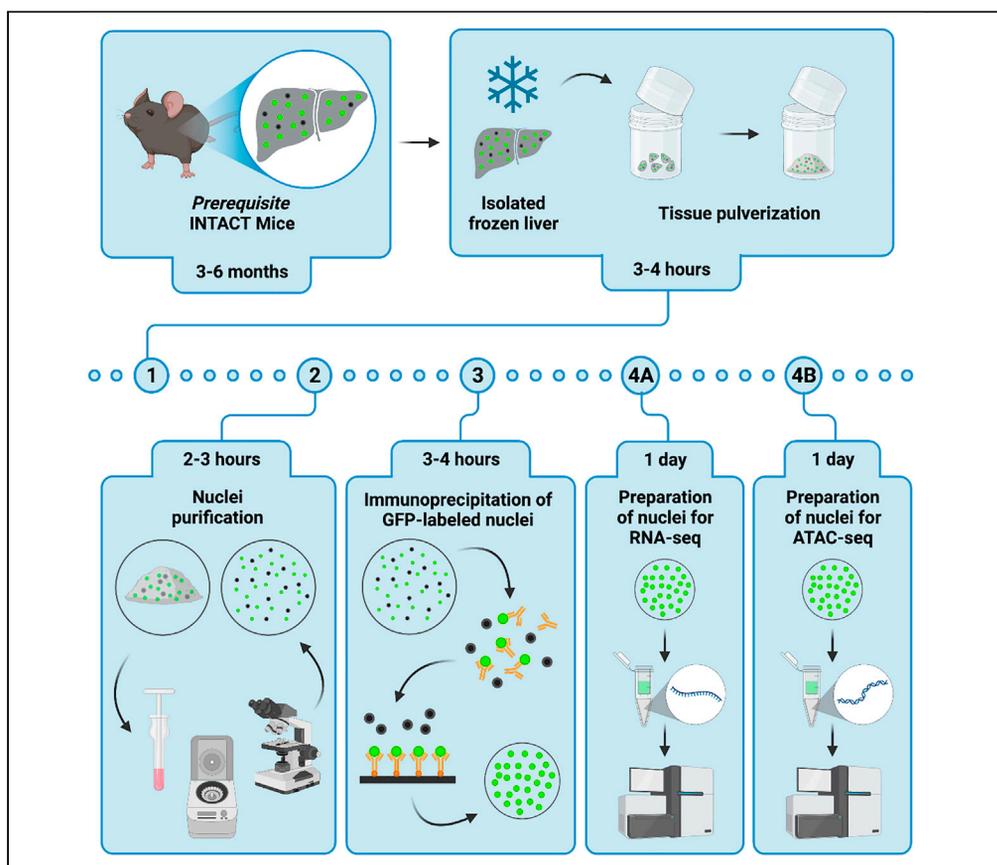


Protocol

Purification of GFP-tagged nuclei from frozen livers of INTACT mice for RNA- and ATAC-sequencing



Isolation of nuclei tagged in specific cell types (INTACT) allows for stress-free and high-throughput analyses of cellular subpopulations. Here, we present an improved protocol for isolation of pure and high-quality GFP-labeled nuclei from frozen livers of INTACT mice, as well as protocols for downstream sequencing analyses. The adaptation to frozen tissue provides a pause point that allows sampling at multiple time points and/or phenotypic characterization of livers prior to nuclei isolation and downstream analyses.

Anne Loft, Stephan Herzig, Søren Fisker Schmidt

anlo@bmb.sdu.dk (A.L.)
sfs@bmb.sdu.dk (S.F.S.)

Highlights

Optimized protocol for isolation of nuclei from frozen livers

Protocol for immunoprecipitation of nuclei tagged in specific cell types (INTACT)

Preparation of nuclear RNA- and ATAC-seq libraries

Nuclei from frozen and fresh livers perform equally in downstream assays

Loft et al., STAR Protocols 2, 100805
September 17, 2021 © 2021
The Author(s).
<https://doi.org/10.1016/j.xpro.2021.100805>



Protocol

Purification of GFP-tagged nuclei from frozen livers of INTACT mice for RNA- and ATAC-sequencing

Anne Loft,^{1,2,3,4,5,6,7,*} Stephan Herzig,^{1,2,3,4} and Søren Fisker Schmidt^{1,2,3,4,5,6,8,*}

¹Institute for Diabetes and Cancer (IDC), Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg 85764, Germany

²Joint Heidelberg-IDC Translational Diabetes Program, Heidelberg University Hospital, Heidelberg 69120, Germany

³Molecular Metabolic Control, Medical Faculty, Technical University Munich, München 80333, Germany

⁴German Center for Diabetes Research (DZD), Neuherberg 85764, Germany

⁵Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense 5230, Denmark

⁶Center for Functional Genomics and Tissue Plasticity (ATLAS), University of Southern Denmark, Odense 5230, Denmark

⁷Technical contact

⁸Lead contact

*Correspondence: anlo@bmb.sdu.dk (A.L.), sfs@bmb.sdu.dk (S.F.S.)

<https://doi.org/10.1016/j.xpro.2021.100805>

SUMMARY

Isolation of nuclei tagged in specific cell types (INTACT) allows for stress-free and high-throughput analyses of cellular subpopulations. Here, we present an improved protocol for isolation of pure and high-quality GFP-labeled nuclei from frozen livers of INTACT mice, as well as protocols for downstream sequencing analyses. The adaptation to frozen tissue provides a pause point that allows sampling at multiple time points and/or phenotypic characterization of livers prior to nuclei isolation and downstream analyses.

For complete details on the use of this protocol, please refer to Loft et al. (2021).

BEFORE YOU BEGIN

The protocol presents an optimized strategy for preparing pure, high-quality nuclei of a certain cellular subpopulation from frozen mouse livers. The protocol relies on the INTACT methodology and generation of so-called INTACT mice that apply a Cre-Lox system to express a tagged version of a nuclear membrane protein allowing for pull-down based affinity purification of genetically labeled nuclei from defined cell populations (Deal and Henikoff, 2010; Mo et al., 2015). INTACT has the advantage over e.g., fluorescence-activated cell sorting-based approaches in that it poses less stress to the cells/nuclei during the isolation, and further that a high number of samples can be handled within a relatively short timeframe.

However, not all experimental setups allow working on fresh tissue preparations; thus, we have optimized the protocol to obtain high-quality nuclei from frozen liver tissue. In the first sections, we describe how to purify hepatocyte nuclei from frozen livers of healthy HEP-INTACT male and female mice of 3–6 months of age, but we have also applied this protocol to obtain hepatocyte nuclei from older mice (6–12 months) with fatty and fibrotic livers as well as myeloid nuclei from livers of MAC-INTACT mice. In the later sections, we outline how these high-quality nuclei easily can be prepared for nuclear RNA-seq, ATAC-seq and proteomics approaches. We further provide examples that the nuclei preparations from frozen tissues perform equally well compared to preparations from fresh tissues in terms of quality, purity and in downstream sequencing applications.



This optimized protocol is therefore well-suited for experiments that do not allow for the use of fresh tissue preparations such as time course experiments and experiments that involve preceding phenotypic characterization such as histology.

Generate HEP INTACT mice

⌚ Timing: 3–6 month

To allow for selective pulldown of the cell type of interest CAG-Sun1/sfGFP reporter mice (B6;129-Gt(ROSA)26Sor^{tm5(CAG-Sun1/sfGFP)Nat/J}) (Mo et al., 2015) should be crossed with specific Cre-driver mice. Specific labeling of hepatocytes can be achieved by crossing the CAG-Sun1/sfGFP reporter mice with Albumin-Cre mice (B6N.Cg-Speer6-ps1^{Tg(Alb-cre)21Mgn/J}) (Postic et al., 1999) to generate so-called HEP-INTACT mice.

Note: We have also generated MAC-INTACT mice with myeloid-specific labeling by crossing the CAG-Sun1/sfGFP reporter mice with LysM-Cre mice (B6N.129P2(B6)-Lyz2^{tm1(cre)lfo/J}) (Clausen et al., 1999). The following protocol also works with MAC-INTACT mice, although a few steps need to be adapted as outlined in the text.

Note: If possible, breed both parental lines as homozygous prior to intercross, to allow for generation of INTACT mice heterozygous for both Cre and for the CAG-Sun1/sfGFP reporter at a 100% rate. This is possible for CAG-Sun1/sfGFP reporter mice as well as for both the abovementioned Albumin-Cre mice and LysM-Cre mice but needs to be assessed for each individual Cre-driver line. Depending on the research question, backcrossing to a defined background may need to be considered. The genetic composition of CAG-Sun1/sfGFP reporter mice available at Jax is approximately 75% B6J and 25% 129SV. Speed congenics can be considered for accelerating backcrossing to a specific desired background.

Note: Specificity and efficacy of the Cre-driver can be assessed using e.g., immunofluorescence prior to proceeding to nuclear isolation and purification. Of note, we observed no labeling of nuclei in the absence of Cre-expression in the liver. Livers of Cre- animals can be used as negative controls in the isolation protocol, particularly for establishing the protocol. However, for larger experiments, nuclei isolated from the whole liver suspension are more cost-efficient controls, and it is further an advantage that these are obtained from the same animals as the purified nuclei of interest.

Prepare buffers and reagents for nuclei purification

⌚ Timing: 1 h

1. Clean all instruments, including grinding jars and dounce homogenizers with cleaning agents for removing RNases (e.g., RNaseZap; Sigma; R2020) and wash with DEPC-treated water before start.
2. Prepare low sucrose buffer (LSB), high sucrose buffer (HSB), wash buffer (WB) and WB, incl. RNasin.
 - a. Filter buffers using a 0.2 μm syringe filter and pre-cool buffers on ice.
3. Prepare nuclear extraction buffer (NUN) if performing protein analyses.
4. Reconstitute lyophilized anti-GFP antibody in 0.5 mL PBS, pH 7.4 to prepare a 0.2 mg/mL stock solution

Note: Reconstituted antibody can be stored for up to 3 months at 4°C with the addition of 2 mM sodium azide.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-GFP antibody <i>Reconstitute lyophilized anti-GFP antibody in 0.5 mL PBS, pH 7.4.</i>	Thermo Fisher Scientific	Cat: G10362; RRID:AB_2536526
Chemicals, peptides, and recombinant proteins		
Invitrogen™ TRIzol™ Reagent	Thermo Fisher Scientific	15596018
cOmplete™, EDTA-free Protease Inhibitor	Roche/Sigma	11873580001
cOmplete™ Mini, EDTA-free Protease Inhibitor	Roche/Sigma	11836170001
RNasin® Plus Rnase Inhibitor	Promega	N2615
Invitrogen™ Dynabeads™ Protein G	Thermo Fisher Scientific	10004D
Gibco™ Trypan Blue Solution, 0.4%	Thermo Fisher Scientific	15250061
DAPI	Sigma	D9542
Sucrose BioReagent	Sigma	S1888
IGEPAL® CA-630	Sigma	56741
DL-Dithiothreitol (DTT)	Sigma	D0632
Spermine tetrahydrochloride, BioReagent	Sigma	S1141
Spermidine, BioReagent	Sigma	S0266
Invitrogen™ 5M NaCl, RNase-free	Thermo Fisher Scientific	AM9760G
Invitrogen™ 2M KCl, RNase-free	Thermo Fisher Scientific	AM9640G
Invitrogen™ 1M MgCl ₂ , RNase-free	Thermo Fisher Scientific	AM9530G
Invitrogen™ Ultrapure™ 1M Tris-HCl, pH 7.5	Thermo Fisher Scientific	15567027
Invitrogen™ UltraPure™ 1 M Tris-HCl Buffer, pH 8.0	Thermo Fisher Scientific	15568025
Invitrogen™ Ultrapure™ Urea	Thermo Fisher Scientific	15505027
Gibco™ 1× PBS, pH 7.4	Thermo Fisher Scientific	10010023
Invitrogen™ Nuclease-Free Water	Thermo Fisher Scientific	4387936
Critical commercial assays		
RNase-Free DNase Set (DNase I and Buffer RDD)	QIAGEN	79254
MinElute Reaction Cleanup Kit	QIAGEN	28204
MinElute PCR Purification Kit	QIAGEN	28004
NEBNext® High-Fidelity 2× PCR Master Mix	New England Biolabs Inc.	M0541S
High Sensitivity DNA Kit	Agilent	5067-4626
VAHTS Stranded mRNA-seq Library Kit for Illumina	Vazyme	NR602
TruePrep™ DNA Library Prep Kit V2 for Illumina® (contains 5× TTBL and TTE Mix V50)	Vazyme	TD501
TruePrep Index Kit V2 for Illumina®	Vazyme	TD202
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific	23225
Deposited data		
INTACT RNA-seq data	This study	GEO:GSE181940
INTACT ATAC-seq data	This study	GEO:GSE181937
Experimental models: organisms/strains		
<i>B6;129-Gt(ROSA)26Sor^{tm5(CAG-Sun1/sfGFP)Nat} / J</i> mice Female and male mice aged 8–26 wks were used for breeding.	JAX	Stock: 021039
<i>B6N.Cg-Speer6-ps1^{Tg(Alb-cre)21Mgn} / J</i> Female and male mice aged 8–26 wks were used for breeding.	JAX	Stock: 018961
<i>B6N.129P2(B6)-Lyz2^{tm1(cre)lfc} / J</i> Female and male mice aged 8–26 wks were used for breeding.	JAX	Stock: 018956
Other		
TissueLyser II	QIAGEN	85300
Grinding Jar Set, Teflon	QIAGEN	69986
DWK Life Sciences Kimble™ Kontes™ Dounce Tissue Grinders	DWK Life Sciences	8853000015

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
100 μ m CellTrics filter unit	Sysmex Deutschland GmbH	04-0042-2318
Invitrogen™ DynaMag™-2 Magnet	Thermo Fisher Scientific	12321D
Eppendorf™ DNA LoBind Tube 1.5 mL / 2 mL	Sigma	EP0030108051 / EP0030108078
EconoSpin Micro Columns	Epoch	3010-250
RPE buffer concentrate	QIAGEN	1018013
AMPure XP Beads	Beckman Coulter	A63881

MATERIALS AND EQUIPMENT

TissueLyser II (QIAGEN) including grinding jar set

This equipment is needed to pulverize frozen liver tissue before nuclei purification, which allows for an easier downstream douncing procedure, especially for fatty or fibrotic liver tissues, ultimately resulting in a higher nuclei recovery. We used Teflon grinding jar sets for the tissue pulverization and they worked impeccably. However, over time the Teflon grinding jars tended to break due to handling in liquid nitrogen and therefore steel grinding jars could be considered instead, although not tested in the current protocol. Furthermore, there are several alternative ways to pulverize frozen liver tissues (Liang et al., 2019), which can be used instead of the suggested method. E.g., for small amounts of tissue, a liquid nitrogen-cooled mortar and pestle may be preferable.

Douncers for tissue homogenization

For tissue homogenization, we used 15 mL glass dounce tissue grinders supplied with a loose pestle for initial sample reduction and a tight pestle to form the final homogenate. The protocol is optimized for initial tissue homogenization using 4 mL low sucrose buffer per 0.5 g of tissue, and we tested that these douncers effectively could homogenize up to at least 1 g of liver tissue. Alternative (smaller) douncers could be considered for douncing smaller amounts of pulverized liver tissue, but we recommend to always test the impact of alternative douncers on the protocol outcome.

Antibodies

Various antibodies may be used for the immunoprecipitation (IP) of the INTACT-labeled nuclei. In this protocol, we applied a monoclonal anti-GFP antibody (Thermo Fisher Scientific; G10362) targeting the two tandem copies of the superfolder GFP tagged to the SUN1 nuclear membrane protein. However, it is also possible to use anti-MYC antibodies targeting the six copies of the MYC epitope on SUN1. During the protocol optimization, we tested various antibodies and they performed differently in terms of yield and purity. Notably, one antibody (anti-MYC tag antibody (Thermo Fisher Scientific; MA1-21316)) led to extensive nuclei aggregation following the pulldown procedure and another (anti-MYC tag antibody (Abcam; ab9106)) appeared to unexpectedly interact with nuclei originating from hepatic stellate cells. Therefore, these antibodies should be avoided, and alternative antibodies should be thoroughly tested before applied in this protocol.

Nanodrop and bioanalyzer for assessment of sample quality

In this protocol, we used the NanoDrop™ 2000 (Thermo Fisher Scientific) to evaluate the purity and concentration of the nuclear RNA; however, alternative similar instruments can easily be used for this purpose. Furthermore, we used the Bioanalyzer 2100 (Agilent) to assess the integrity of the RNA and to quantify libraries during the sequencing preparations. Alternative instruments may be used here as well.

Buffers for nuclei isolation

The following recipes are sufficient for nuclei preparation from 12 frozen mouse livers.

Low sucrose buffer (LSB)

Reagent (stock conc)	Final concentration	Amount
Sucrose	250 mM	8.56 g
Tris-HCl, pH 7.5 (1 M)	15 mM	1.5 mL
MgCl ₂ (1 M)	5 mM	0.5 mL
KCl (2 M)	25 mM	1.25 mL
Spermidine (1 M)	0.5 mM	50 μL
Spermine (1 M)	0.15 mM	15 μL
DTT (1 M)	1 mM	100 μL
RNasin (only for RNA analyses)	15 U/mL	150 μL
EDTA-free protease inhibitor cocktail (PIC)		2 tablets
Nuclease-Free Water		Up to 100 mL
Total	NA	100 mL

High sucrose buffer (HSB)

Reagent (stock conc)	Final concentration	Amount
Sucrose	2 M	27.36 g
Tris-HCl, pH 7.5 (1 M)	15 mM	0.6 mL
MgCl ₂ (1 M)	5 mM	0.2 mL
KCl (2 M)	25 mM	0.5 mL
Spermidine (1 M)	0.5 mM	20 μL
Spermine (1 M)	0.15 mM	6 μL
DTT (1 M)	1 mM	40 μL
RNasin (only for RNA analyses)	15 U/mL	60 μL
EDTA-free PIC		1 tablet
Nuclease-Free Water		Up to 40 mL
Total	NA	40 mL

Wash buffer (WB)

Reagent (stock conc)	Final concentration	Amount
IGEPAL CA-630 (5%)	0.35%	17.5 mL
Sucrose	250 mM	21.4 g
Tris-HCl, pH 7.5 (1 M)	15 mM	3.75 mL
MgCl ₂ (1 M)	5 mM	1.25 mL
KCl (2 M)	25 mM	3.125 mL
Spermidine (1 M)	0.5 mM	125 μL
Spermine (1 M)	0.15 mM	37.5 μL
DTT (1 M)	1 mM	250 μL
EDTA-free PIC		2 tablets
Nuclease-Free Water		Up to 250 mL
Total	NA	250 mL

WB, incl. RNasin

Reagent (stock conc)	Final concentration	Amount
WB (prepared from above)		30 mL
RNasin	15 U/mL	45 μL
Total	NA	30 mL

Note: DTT, spermine, spermidine, RNasin, and protease inhibitor should always be added right before the start of the experiment, the remaining buffer components can be prepared in advanced and kept for a maximum of 2 weeks at -20°C .

Note: The sucrose (especially in HSB) might need some time to solubilize. It is possible to use e.g., a roller mixer to facilitate the process. Further, the resuspension of the PIC tablet in HSB can also be facilitated using a roller mixer.

Note: 1M DTT, spermine, and spermidine stock aliquots can be prepared in advance and stored at -20°C . Do not re-freeze a thawed aliquot.

Note: A 5% IGEPAL solution can be prepared beforehand and stored for several months at 20°C – 25°C .

Note: RNasin is only needed for experiments intended for RNA analyses.

NUN extraction buffer		
Reagent (stock conc)	Final concentration	Amount
Tris-HCl, pH 8 (1 M)	50 mM	50 μL
Urea	2 M	120 mg
NaCl (5 M)	600 mM	120 μL
DTT (1 M)	1 mM	1 μL
mini EDTA-free PIC		1 tablet
Nuclease-Free Water		Up to 1 mL
Total	NA	1 mL

Note: Add water as the last component, since urea will not dissolve easily in water.

Note: NUN extraction buffer should be used within one day

Alternatives: It should be possible to replace all reagents listed in the [key resources table](#) with equivalent items from other suppliers, but this has not been tested.

STEP-BY-STEP METHOD DETAILS

Pulverize frozen liver extracts

⌚ **Timing:** 3–4 h for 12 tissue preparations

In our experience, the best preparations of nuclei from snap-frozen liver samples are obtained by pulverizing the tissue into fine powder before starting the purification of the nuclei.

1. Isolation of mouse liver tissues.
 - a. Sacrifice the mouse humanely following an approved ethical protocol.
 - b. Pin down the mouse and open the abdominal cavity.
 - c. Isolate all liver lobes using scissors and forceps.
 - d. Cut the liver lobes into smaller pieces (approx. 5×5 mm) and wash liver pieces in $1\times$ PBS.
 - e. Snap-freeze liver pieces in liquid nitrogen.

⏸ **Pause point:** At this point, liver tissue pieces can be stored at -80°C for later downstream processing allowing for e.g., initial histological assessment of the liver.

2. Grinding of liver tissue.
 - a. Unscrew the lid of the grinding jar and pre-chill the jar, lid, and the grinding ball in liquid nitrogen.
 - b. Put the grinding ball back into the jar, add the liver pieces into the jar and close the lid firmly.

- c. Grind the liver tissue at 30 Hz for 1 min at the TissueLyser until the tissue is completely pulverized.
- d. Carefully transfer the pulverized tissue into e.g., a 15 mL conical tube and be sure to scrape of remaining pulverized tissue remnants from the side of the jar and from the grinding ball.

△ **CRITICAL:** It is extremely important that all items are kept very cold during tissue handling. It might be necessary to wear extra protection of your hands due to the handling of these very cold items.

▣ **Pause point:** At this point, the pulverized tissues can be stored at -80°C for later downstream processing. In order to have sufficient time for performing the following steps (i.e., isolation and purification of GFP-labeled nuclei as well as the first steps in the preparation of the nuclei for sequencing) within a working day, we normally pause the protocol at this point.

Isolation of nuclei from pulverized liver tissue

⌚ **Timing:** 2–3 h for 12 nuclei preparations

The following steps describe how to purify high-quality nuclei from pulverized liver tissue using a glass dounce homogenizer.

3. Homogenize the liver (see [troubleshooting 1](#)).
 - a. Precool centrifuges to 4°C .
 - b. Resuspend ~ 0.5 g pulverized tissue in 4 mL ice-cold $1\times$ PBS by tapping e.g., in a 5 mL conical tube.

Note: If processing livers with high fat content, the nuclei yield per gram of liver will be lower and a greater amount of pulverized liver tissue can be used as starting material. We have tested that the 15 mL douncer can hold up to approx. 1 g of tissue using 8 mL LSB and still perform equally well. However, it is important not to overload the douncer with more than 1 g of pulverized liver (see [troubleshooting 1](#)). Then rather perform multiple rounds of douncing and combine before proceeding with the IP.

- c. Centrifuge at $2,000\times g$ for 3 min at 4°C .
- d. Remove supernatant and resuspend pellet in 4 mL LSB.
- e. Transfer to douncer and dounce 10 times with loose pestle.
- f. Add 300 μL of a 5% IGEPAL solution to make the solution $\sim 0.35\%$ IGEPAL, mix tubes and let stand at 4°C for 3–5 min.
- g. Dounce 5 times more with tight pestle.
- h. Filter the homogenate through a 100 μm CellTrics filter unit on a 15 mL canonical tube.
- i. Wash the douncer with 1 mL LSB and add through the filter to homogenate in step h.
- j. Spin filtered homogenate at $600\times g$ for 10 min at 4°C .
- k. Resuspend pellet in $9\times$ volume of HSB. Mix by pipetting.
- l. Aliquot up to 1.8 mL in 2 mL tubes.

Note: Normally $9\times$ volume equals to 0.9–1.8 mL HSB per sample. If more than 1.8 mL is needed per sample divide the volume into two 2 mL tubes.

Note: It is recommended to double check that the nuclei pellets have gone completely into suspension. If not, mix samples again by pipetting right before spinning.

- m. Spin 15 min at $15,000\times g$ at 4°C .
- n. Carefully remove as much supernatant as possible.
- o. Resuspend pellets in total 1 mL WB, incl. RNasin per sample.

Note: After pelleting the nuclei in HSB, these might be hard to resuspend and need to be pipetted extensively, but still carefully, for complete resuspension.

Note: The dense sucrose cushion is recommended to remove debris and provide pure and intact nuclei. To avoid transferring the debris on the tube sides, we recommend resuspending first in 500 μ L WB, incl. RNasin, transfer this volume to a new tube and then add additional 500 μ L WB, incl. RNasin.

△ CRITICAL: Carry out above steps on ice or in a cold room at 4°C.

4. Inspection and counting the number of whole liver nuclei (see [Figures 1A and 1B](#), [expected outcomes](#), and [troubleshooting 2 and 3](#)).
 - a. Take out 10 μ L aliquot, dilute it 1:20 in WB and mix 1:1 with Trypan blue.
 - b. Inspect and count the nuclei under a brightfield microscope using a 10–20 \times objective.
 - c. Dilute the nuclei suspension to 10 mill nuclei per mL in WB, incl. RNasin.
 - d. Collect 15 mill nuclei (1.5 mL) for IP of hepatocyte nuclei.

Optional: Collect 1 mill nuclei for whole liver nuclei RNA-seq and 25,000 for whole liver nuclei ATAC-seq and store on ice until further processing. These whole liver nuclei can in the end be used for control purposes, e.g., for determining enrichment/depletion of marker gene expression compared to the bead-bound nuclei by qPCR or RNA-seq.

△ CRITICAL: An appropriate counting of the nuclei is important for the downstream processes. We recommend counting nuclei manually using a standard hemocytometer. Alternatively, the nuclei can be counted using an automated cell counter. Since the absolute nuclei numbers often vary between the different methods, it is important to use the same strategy for each experiment.

Note: Nuclei of high quality will appear round and intact with well-defined edges under the brightfield microscope at 40–60 \times magnification, whereas nuclei of poor quality lose their intactness and begin to show evidence of membrane blebbing (for examples of nuclei with different quality refer to <https://kb.10xgenomics.com/hc/en-us/articles/360020348651-How-can-I-assess-the-quality-of-my-nuclei-for-Single-Cell-ATAC-or-Single-Cell-Multiome-ATAC-GEX-Sequencing->).

Note: For other conditions or types of labeled nuclei, the number of input nuclei can be scaled proportionally to the fraction of GFP+ nuclei. For example, when purifying GFP-labeled myeloid nuclei originating from frozen livers of healthy MAC INTACT mice (consisting of 7%–10% GFP+ nuclei), we used approximately 80 mill whole liver nuclei as input for the IP but kept the reaction volume and amount of antibody and Dynabeads as described below.

Immunoprecipitation of GFP-labeled nuclei

⌚ **Timing:** 3–4 h for 12 nuclear preparations

The following steps describe how to pull down GFP-labeled nuclei from purified whole liver nuclei.

5. Washing of Dynabeads.
 - a. Aliquot and wash 100 μ L of Protein G Dynabeads (per sample) 2 \times with 800 μ L Wash Buffer in a 2 mL tube on a DynaMag™-2 Magnet.

Optional: Protein G Dynabeads can also be batch-washed in 15 mL tubes using e.g., a DynaMag™-15 magnet (ThermoFisher Scientific; 12301D). To have enough beads for 12 nuclear preparations, use 1350 μ L beads and 12 mL WB.

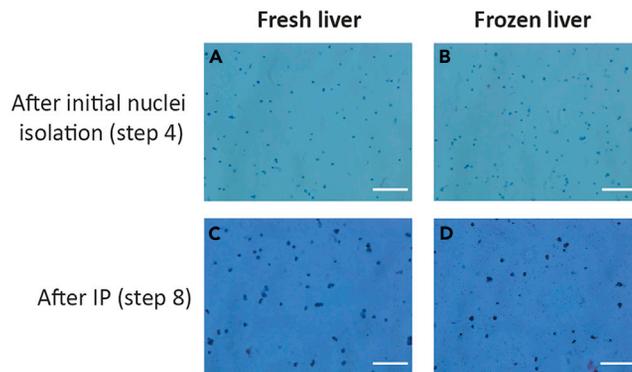


Figure 1. Nuclei purification from pulverized fresh and frozen liver tissue

(A and B) Nuclei were isolated from (A) fresh and (B) frozen livers, stained with Trypan blue and investigated using a microscope. Scale bars represent 200 μm .

(C and D) Nuclei were immunoprecipitated using anti-GFP antibody and magnetic Dynabeads, stained with Trypan blue and investigated using a microscope. Scale bars represent 100 μm .

- b. Resuspend beads in 100 μL per sample of WB, incl. RNasin.
 - c. Pipette 20 μL of beads into new 2 mL tube (labeled "Preclear").
6. Pre-clearing of nuclei.
 - a. Transfer the nuclei suspension into the preclear tube and mix.
 - b. End-to-end rotate at 4°C for 10–15 min.
 7. IP of GFP-labeled nuclei.
 - a. While nuclei sample is preclearing, prepare 2 mL tubes with 3 μg of anti-GFP antibody (i.e., 15 μL) and 200 μL WB, incl. RNasin in each tube.
 - b. Place precleared sample on magnet for 4–5 min.
 - c. While standing on the magnet, pipet the supernatant from the precleared sample into the tube with antibody.
 - d. End-to-end rotate at 4°C for 30 min.
 - e. Resuspend the remaining 80 μL of washed beads and add to tube with antibody and nuclei.
 - f. End-to-end rotate at 4°C for 20 min.
 - g. Place sample on magnet for 4–5 min.
 - h. While standing on the magnet, completely remove the supernatant.

Optional: the supernatant contains the nuclei that was not captured in the IP reaction (primarily non GFP-labeled nuclei) and can be saved as controls for later analyses. However, from frozen liver tissues this fraction also contains a considerable number of unbound GFP+ nuclei.

- i. Remove samples from magnet; add 1.8 mL of WB and let sit for 30 s.

Note: RNasin is omitted from the WB during the washes to reduce costs.

- j. Pipette extensively (but carefully) up and down to resuspend. After initial resuspension, pipette up and down an additional 5 times.
- k. Repeat step g-j twice.
- l. Finally take the tube of the magnet, add 1 mL WB, incl. RNasin and let sit over the nuclei for 2 min before resuspending.

△ CRITICAL: carry out all steps on ice or in the cold room at 4°C, including the steps involving a magnet or an end-to-end rotator.

8. Inspection and counting of bead-bound nuclei (see also [Figures 1C and 1D](#), [expected outcomes](#) and [troubleshooting 4](#)).
 - a. Take out 10 μ L aliquot of bead-bound nuclei, dilute it 1:5 in WB and mix 1:1 with Trypan blue.
 - b. Count the number of nuclei using a brightfield microscope using a 10–20 \times objective.
 - c. Calculate the fraction of recovered nuclei by comparing the number of bead-bound nuclei with the number of whole liver nuclei initially added to the IP reaction (i.e., 15 mill nuclei).

Optional: Determine purity and yield of GFP+ nuclei (see [Figure 2](#), [expected outcomes](#) and [troubleshooting 5](#))

- d. Add 1 μ L DAPI to a 10 μ L aliquot of bead-bound and whole liver nuclei (diluted to approx. 7500 nuclei / μ L)
- e. Count 100–200 nuclei with a fluorescence microscope equipped with appropriate filters for detecting GFP and DAPI using a 10–20 \times objective.
- f. Quantify the number of bead-bound GFP+ nuclei vs. DAPI-stained nuclei in both samples.
- g. Determine the purity by considering the fraction of GFP+ nuclei vs total DAPI-stained nuclei in the bead-bound sample.
- h. To determine the yield, compare the actual number of recovered GFP+ nuclei with the expected number of recovered GFP+ nuclei, which can be calculated as follows: Expected # of GFP+ nuclei = Total # of nuclei added to IP \times % GFP+ nuclei in the original whole liver sample.

Note: The unbound Dynabeads will result in some green auto fluorescence; however, since the beads are much smaller than the complexes of nuclei and beads, these should be easily distinguishable when counting.

Note: If handling a larger number of nuclei preparations, the easier option for determining purity and yield will be to take some images using the fluorescence microscope coupled to a computer and do the quantification with e.g., ImageJ (<https://imagej.nih.gov/ij/download.html>) at a later time point.

▣▣ Pause point: At this point, it is possible to save (extra) nuclei for later processing or alternative applications by using a slow-freezing protocol. For further details, please refer to [Corces et al., 2019](#); [Corces et al., 2017](#), which describe how purified nuclei can be frozen and subsequently used for ATAC-seq and other downstream applications without adversely affecting data quality. However, for the current protocol, we recommend continuing with the procedure until nuclei are stored in TRIzol (for RNA analyses), in NUN buffer (for protein analyses) or until the transposition reaction followed by the DNA purification has been performed (for ATAC-seq analyses).

Downstream applications

In the following sections we describe three different downstream applications for the purified nuclei. The different procedures can be performed independently, and in any order. However, since the procedure for preparation of nuclei for RNA-seq has a safe pause point at an early step in the procedure, we normally begin with this procedure.

Prepare nuclei for RNA-seq

⌚ **Timing:** ~20 min until nuclei are stored in TRIzol, 2–3h for 12 complete RNA purifications, and 1 additional day if preparing RNA for sequencing

The following steps describe how to isolate high-quality RNA from bead-bound and whole liver nuclei.

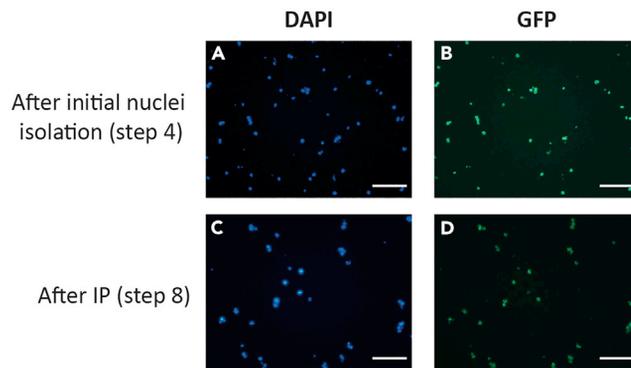


Figure 2. GFP+ nuclei detection in whole liver and bead-bound nuclei preparations

(A and B) Whole liver and (C and D) bead-bound nuclei were isolated from frozen livers, stained with DAPI and subsequently (A and C) DAPI and (B and D) GFP signals were investigated using a fluorescence microscope. Scale bars represent 100 μm .

9. Harvest nuclei for RNA isolation.

a. Whole liver nuclei.

- i. Spin down 1 mill nuclei at $600\times g$ for 5 min at 4°C .
- ii. Remove supernatant and leave 20–30 μL WB, incl. RNasin behind to resuspend pellet again.
- iii. Vortex nuclei to resuspend, add 500 μL of TRIzol and vortex until nuclei are completely dissolved.

b. For bead-bound nuclei.

- i. Take a volume out corresponding to 1 mill nuclei.
- ii. Use a magnet to remove supernatant leaving 20–30 μL WB, incl. RNasin behind to resuspend pellet again.

△ CRITICAL: Failure to properly resuspend the nuclei pellet will cause nuclei to remain aggregated when adding TRIzol.

- iii. Briefly spin down to collect nuclei and WB in the bottom of the tube.
- iv. For one tube at a time vortex, immediately add 500 μL of TRIzol and vortex until completely dissolved

10. RNA column purification procedure

- a. Add 0.2 volume (100 μL) of chloroform and vortex vigorously for 30 s to mix phases.
- b. Incubate 15 min at 20°C – 25°C and spin at $10,000\times g$ for 10 min at 4°C .
- c. Transfer the upper phase (approx. 200 μL) to a 1.5 mL tube and place on ice.

△ CRITICAL: Do not touch the interphase!

- d. Add 0.6 volume (120 μL) 96% ethanol, briefly vortex, and briefly spin down.
- e. Load the indicated volume onto an EconoSpin microcolumn, spin $13,000\times g$ for 30 s at 20°C – 25°C .

Note: Close the lid on the column gently.

- f. Wash the column once with 500 μL diluted RPE buffer at 20°C – 25°C . Spin at $13,000\times g$ for 30 s at 20°C – 25°C and discard the flow through.

△ CRITICAL: Confirm that ethanol has been added to the RPE buffer before use.

Note: A multistep pipette can be used if handling multiple samples.

- g. Make a DNase I mix, by combining 10 μL DNase I stock solution with 70 μL Buffer RDD. Mix by gently inverting the tube and shortly spin down the tube before use.

Note: It is possible to prepare a DNase mastermix if multiple samples are processed.

- h. Add the DNase I mix (80 μL) directly to the microcolumn membrane, and place on the bench-top (20°C–30°C) for 15 min.
- i. Add 500 μL diluted RPE buffer to the microcolumn with the DNase I incubation mix. Close the lid gently, and centrifuge at 13,000 $\times g$ for 30 s at 20°C–25°C to wash the spin column membrane free of DNaseI.
- j. Discard the flow-through and collection tube.
- k. Place the microcolumn in a new 2 mL collection tube and wash the microcolumn one additional time with 500 μL diluted RPE buffer.
- l. Spin at 13,000 $\times g$ for 30 s at 20°C–25°C and discard the flow through.
- m. Place the microcolumn back in the collection tube and spin dry at 13,000 $\times g$ for 2 min at 20°C–25°C.
- n. Transfer the column to a new 1.5 mL tube and incubate the column with lid open for 5–10 min at 20°C–25°C to completely evaporate residual ethanol.

△ CRITICAL: Be careful not to touch the sides of the microcolumn while transferring the column between tubes!

- o. Elute in 16 μL RNase-free water by adding to the center of the column and incubate for 2 min at 20°C–25°C.
- p. Spin at 9,000 $\times g$ for 2 min at 20°C–25°C and collect eluate.

Note: If lid breaks, transfer the eluate to a new 1.5 mL tube.

▮▮▮ Pause point: Proceed for downstream applications or store at –80°C.

11. Measure RNA concentration
 - a. Measure the RNA concentration and quality on 1 μL of sample on the Nanodrop.

Note: The ratios A260/A280 and A260/A230 should be ≥ 1.8 and ideally ≥ 2 .

Note: If 1 mill nuclei are used for the RNA-analyses, the expected RNA concentrations are >50 ng/ μL . A lower number of nuclei can be used for RNA analyses depending on the downstream purposes.

Optional: Determine RNA integrity.

It is normally recommended to measure the quality of the RNA e.g., using the RNA integrity number (RIN) computed by the Bioanalyzer. However, since the RIN score is based on the ratio of the 18S to 28S ribosomal RNA molecules this is not a suitable measure for nuclear RNA. In fact, we usually obtained RIN scores around 2–3 for the nuclear RNA, which still performed impeccably in the RNA sequencing preparations. Instead, we used the Bioanalyzer profiles to manual inspect the RNA integrity, since we observed a clear difference in the amount of degraded RNA with the presence of RNasin in the buffers (Figures 3A and 3B).

12. Prepare RNA for sequencing
 - a. 100–200 ng of RNA can be prepared for sequencing using the VAHTS Stranded mRNA-seq Library Kit for Illumina according to the manufacture’s recommendations (https://v4.cecdn.yun300.cn/100001_2005225118%2FNR602.pdf), except that no enrichment of mRNA was performed. Instead, 5 μL of (diluted) RNA was added directly to 13.5 μL of Frag/Prime Buffer

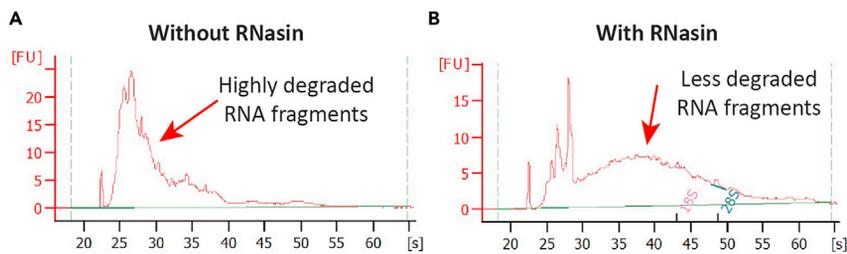


Figure 3. RNasin in INTACT buffers prevents degradation of nuclear RNA

Nuclei were isolated and immunoprecipitated without or with the presence of RNasin in the buffers, as indicated in the protocol. RNA was subsequently column-purified, and RNA integrity was assayed using a Bioanalyzer 2100. (A) When RNasin was not added to the buffers, the bioanalyzer run showed traces of highly fragmented RNA. (B) When RNasin was added to the buffers, the RNA became less degraded. Note that peaks corresponding to 18S and 28S rRNA fragments are absent from the nuclear RNA trace and therefore the RNA integrity score is not a reliable measure for assessing quality of nuclear RNA.

(1.13 in the original manual) to fragment the RNA before proceeding with the cDNA synthesis.

Note: A lot of manufacturers offer kits for preparation of RNA for sequencing and can be used as alternatives here.

Prepare nuclei for ATAC-seq

⌚ Timing: 2 h for 12 transpositions reactions, 1 additional day if preparing genomic DNA for sequencing.

The following steps describe how to perform a Tn5 transposase reaction (Buenrostro et al., 2015) on purified bead-bound and whole liver nuclei and subsequently prepare the DNA for sequencing. In the following, we enrich specifically for DNA fragments in nucleosome-free regions (NFR) since we are particularly focused on the accessible regions and transcription factor binding events in these regions. If also interested in mono-, di- and multi-nucleosome fragments the protocol should be adjusted accordingly.

13. Perform transposition reaction.
 - a. Thaw 5× TTBL from the TruePrep DNA Library Prep Kit V2 at 20°C–25°C. Mix by inverting.
 - b. For whole liver nuclei.
 - i. Spin down 25,000 nuclei at 600×g for 5 min at 4°C.
 - ii. Remove supernatant completely.
 - c. For bead-bound nuclei.
 - i. Take a volume out corresponding to 25,000 nuclei.
 - ii. Use a magnet to remove supernatant completely.
 - d. Prepare a transposition reaction mastermix (50 μL per sample):

Component	Amount
5 X TTBL buffer	10 μL
TTE Mix V50	3.5 μL
Nuclease-free water	36.5 μL
Total	50 μL

- e. Resuspend the nuclear pellet directly in the transposition reaction mixture by gently pipetting for 20 times.

f. Put the tube in the thermal block at 37°C for 30 min at 1000×rpm with heat lid on at 105°C.

14. Purify DNA fragment

a. Add 5 volumes (250 µL) of Buffer ERC and 5 µL Na-acetate (3 M, pH = 5.2) from the MinElute Reaction Cleanup Kit to the transposition reaction mix.

△ **CRITICAL:** Check that the color of the mixture is yellow similar to the Buffer ERC.

b. Mix by vortexing and incubate for 5 min at 20°C–25°C.

c. Transfer the cleaned-up reaction mix by:

i. For bead-bound nuclei, place mixture on the magnet and transfer supernatant to a MinElute column.

ii. For whole liver nuclei, transfer the whole reaction mix to a MinElute column.

d. Proceed with Qiagen MinElute Reaction Cleanup according to manufactures recommendations (<https://www.qiagen.com/us/resources/download.aspx?id=8f6b09b2-6dcd-4b55-bb4a-255ede40ca3b&lang=en>) and elute DNA in 21 µL elution buffer.

▮▮ **Pause point:** The samples can be stored at –20°C until ready for amplification.

Note: The transposition reaction reagents from Vazyme are not compatible with the OMNI ATAC reagents described in (Corces et al., 2017).

15. PCR Enrichment and purification

a. Place the sterile PCR tube on ice and add each component in order:

Component	Amount
Transposed DNA	10 µL
2× NEBNext PCR Master Mix	12 µL
N5XX	1 µL
N7XX	1 µL
Total	24 µL

Note: The TruePrep Index Kit from Vazyme provides 8 kinds of N5XX and 12 kinds of N7XX. Choose according to the number of samples and strategy of Index selection. Due to adaptor hopping, unique combinations of N5XX and N7XX should be selected for each sample, if possible.

Note: We recommend running the sample index PCR step using only half of the transposed DNA sample volume. The remaining sample volume can be stored at –20°C up to a month and may be used for another round of sample index PCR if necessary.

b. Place the PCR tube in the PCR instrument.

c. Set heat lid on (105°C) and run the PCR as follows:

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial chain displacement reaction	72°C	5 min	1
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	15 s	11–13 cycles
Annealing	60°C	30 s	
Extension	72°C	1 min	
Hold	4°C	Infinite	

Note: For most preparations, 11 cycles of PCR are sufficient. If there is not sufficient DNA for sequencing, use the other half of the transposed DNA volume to perform another round of sample index PCR with more cycles.

- d. Perform cleanup of libraries after the PCR using MinElute PCR Purification Kit according to the manufacturer's recommendations (<https://www.qiagen.com/us/resources/download.aspx?id=8f6b09b2-6dcd-4b55-bb4a-255ede40ca3b&lang=en>) and elute in 21 μ L elution buffer.

△ CRITICAL: Use different reagents for pre- and post-amplification samples and consider working in designated pre- and post-amplification areas to avoid contamination with amplified PCR-products.

16. Examine PCR libraries and perform size fractionation of pooled libraries.
 - a. Run 1 μ L of each library (diluted 1:3 in water) using the High Sensitivity DNA kit according to the manufacturer's recommendations (https://www.agilent.com/cs/library/usermanuals/Public/G2938-90321_SensitivityDNA_KG_EN.pdf) (Figures 4A and 4B and troubleshooting 6).
 - b. Estimate the molarity of each library in the desired size range and pool samples for sequencing.
 - c. Fill up the volume to a total 50 μ L with nuclease-free water before the next step.
 - d. Mix AMPure® XP beads by vortexing and add 32.5 μ L (0.65 \times) of beads to the purified PCR products (50 μ L). Mix thoroughly by gently pipetting for 10 times and incubate at 20°C–25°C for 5 min.
 - e. Place the reaction tube on the magnetic stand after a brief centrifugation. Let the tube rest until the solution completely clarifies (around 5 min)
 - f. Transfer the supernatant carefully to a new tube and discard the beads.
 - g. Mix AMPure® XP beads by vortexing and add 55 μ L (1.1 \times) of beads to the supernatant. Mix thoroughly by gently pipetting for 10 times and incubate at 20°C–25°C for 5 min.
 - h. Place the reaction tube on the magnetic stand after a brief centrifugation. Let the tube rest until the solution completely clarifies
 - i. Remove the supernatant carefully and store the beads.
 - j. Add 200 μ L of fresh prepared 80% ethanol to wash the beads. Incubate at 20°C–25°C for 30 s and discard the supernatant. Repeat for a total of two washes.
 - k. Open the tube lid and air-dry the beads at 20°C–25°C for 5 min.

△ CRITICAL: Do not overdry the beads.

Note: Keep the tube on the magnetic stand while washing and air-drying

- l. Remove the tube from magnetic stand and add 20 μ L of nuclease-free water to elute the DNA. Mix by vortexing or gently pipetting.
- m. Place the reaction tube in the magnetic frame after a brief centrifugation. Let the tube rest until the solution completely clarifies.
- n. Carefully remove 18 μ L of supernatant to a new tube

▮▮ Pause point: The size selected libraries can be stored at –20°C until ready for library quality control.

- o. Run 1 μ L of the pooled size selected library (diluted 1:3 in H₂O) using the High Sensitivity DNA kit according to the manufacturer's recommendations (https://www.agilent.com/cs/library/usermanuals/Public/G2938-90321_SensitivityDNA_KG_EN.pdf) and estimate the molarity (Figures 4C and 4D).

Prepare nuclei for protein analyses

⌚ Timing: 2–3 h for 12 preparations

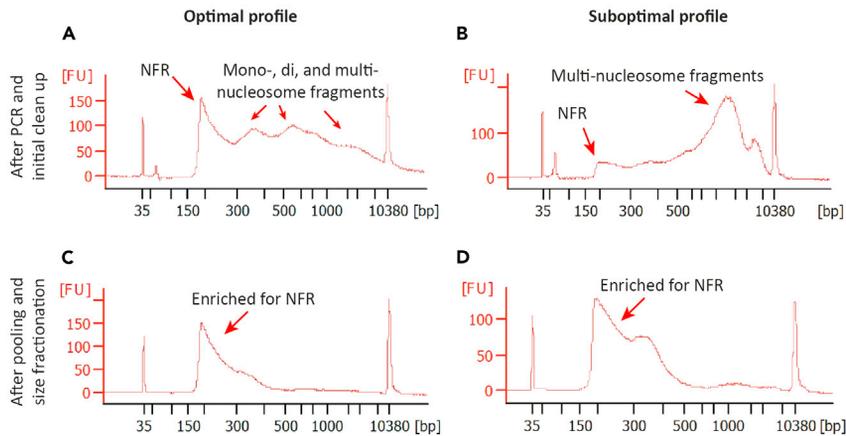


Figure 4. Optimal and suboptimal ATAC-seq libraries

ATAC-seq library quality and quantity were assayed using a Bioanalyzer 2100 (A and B) on the individual libraries after the PCR and initial clean up and (C and D) on the pooled libraries after size fractionation. The peaks of the DNA trace display the periodicity of the chromatin structure representing nucleosome-free regions (NFR), as well as mono-, di-, and multi-nucleosome fragments. (A and B) Examples of DNA traces where (A) a high number of DNA fragments are present in the peak corresponding to the NFR and (B) most DNA represents multi-nucleosome fragments. (C and D) Following pooling of individual libraries and subsequent size fractionation, a further enrichment of DNA fragments in NFR can be achieved, even for suboptimal libraries as observed in B (see also [troubleshooting 6](#)).

17. Harvest of protein.

- Spin down 2 mill nuclei at $600\times g$ for 5 min (whole liver nuclei) or use a magnet (bead-bound nuclei).
- Completely remove all the supernatant.
- Resuspend pellet in 20 μL WB, then add an equal volume of 20 μL NUN extraction buffer and pipet extensively up and down.

Note: After 3–4 rounds of pipetting the solution starts to clump.

- Vortex, spin down shortly and incubate on ice for 20 min.
- Spin 10 min at $21,000\times g$ at 4°C .
- Transfer supernatant to a new tube.
- Take a small aliquot out for determination of the protein concentration.

Pause point: The nuclear extracts can be stored at -80°C until ready for downstream applications.

- Determine nuclear protein concentration using e.g., the BCA Protein Assay Kit according to the manufacturer's recommendations (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011430_Pierce_BCA_Protein_Asy_UG.pdf).

Note: 2 mill hepatocyte nuclei yield around 40 μg protein using this procedure and therefore the expected protein concentration should be around 1 $\mu\text{g}/\mu\text{L}$. If more protein is needed for the downstream applications, the number of input nuclei should be adjusted accordingly.

EXPECTED OUTCOMES

From 0.5 mg of pulverized frozen liver derived from healthy male and female mice aged 3–6 months, we recovered in the range of 60–80 mill nuclei following the initial nuclei purification. The number of nuclei obtained per mg decreased significantly when handling livers from obese mice with fatty livers.

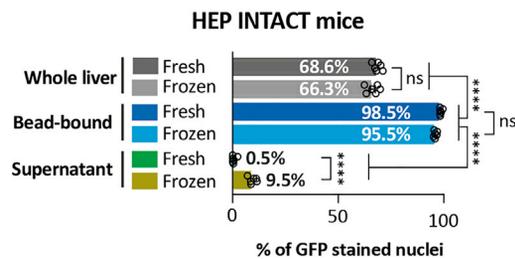


Figure 5. Comparison of INTACT preparations from fresh and frozen liver tissues

Percentage of GFP+ nuclei in the whole liver, as well as in the bead-bound and supernatant fractions from fresh or frozen liver samples (n=6). Colored bars with percentage number represent mean and vertical lines indicate \pm SEM. 2-way ANOVA with post-hoc Tukey's multiple comparison test as indicated; Statistical significance is indicated by ****p<0.0001, ns = not significant.

Using healthy livers obtained from HEP-INTACT mice, we found that around 70% of the total number of isolated nuclei was GFP+ (Figure 5). When purifying labeled nuclei using the indicated anti-GFP antibody, we typically recovered 50%–70% of the GFP+ nuclei, presumably due to some loss of nuclei in the washing steps. Importantly, we observed a comparable high purity (> 95%) of nuclei recovered from fresh and frozen livers (Figure 5). Of note, we found some GFP+ nuclei in the supernatant following the IP when purifying nuclei from frozen liver tissue (Figure 5). We believe this is due to incomplete exposure of the epitope recognized by the antibody because of incomplete removal of the outer nuclear membrane when isolating nuclei from frozen livers.

Using frozen livers from healthy MAC-INTACT mice, which consist of 7%–10% GFP+ nuclei, we typically recovered around 70% of the GFP+ nuclei and achieved a purity of 90%–95%.

We further tested that downstream sequencing assays (i.e., RNA and ATAC-seq) worked equally well for INTACT preparations from fresh and frozen liver isolated from HEP-INTACT mice subjected to a 16 h overnight fast (Figure 6). Here, principal component analysis of the nuclear RNA-seq data demonstrated that GFP+ nuclei isolated from fresh and frozen livers colocalize and separate strongly from GFP- nuclei obtained from fresh livers (Figure 6A). Furthermore, clustering analyses of ATAC-seq data from the GFP+ nuclei fractions showed that nuclei from fresh and frozen livers clustered together both in the fed and in the fasting conditions (Figure 6B). Importantly, for both differentially expressed genes and differentially accessible genomic sites (fresh versus frozen) we found a strong correlation for GFP+ nuclei from fresh and frozen livers (Figures 6C and 6D). See Loft et al., 2021 for details on data analyses.

LIMITATIONS

The nuclei isolation protocol presented here has not been tested for compatibility with single cell approaches; however, it is likely that the Dynabead-based enrichment of nuclei is not compatible with e.g., 10x Chromium Controller system due to the interference of the magnetic Dynabeads with single cell partitioning. Alternative magnetic beads, e.g., Miltenyi Biotec Microbeads, should however be fully compatible with the 10x single cell systems and may be implemented in the current protocol as a replacement of Dynabeads, if single cell analyses are required.

TROUBLESHOOTING

Problem 1

Liver tissue is not homogenized after the suggested number of strokes (step 3)

Potential solution

Since the protocol starts with pulverized tissue, 5–10 strokes with each pestle will normally be sufficient. However, the amounts of strokes may vary depending on the douncers used and might need to be further optimized. In our hands, pulverized fibrotic livers did not require additional strokes

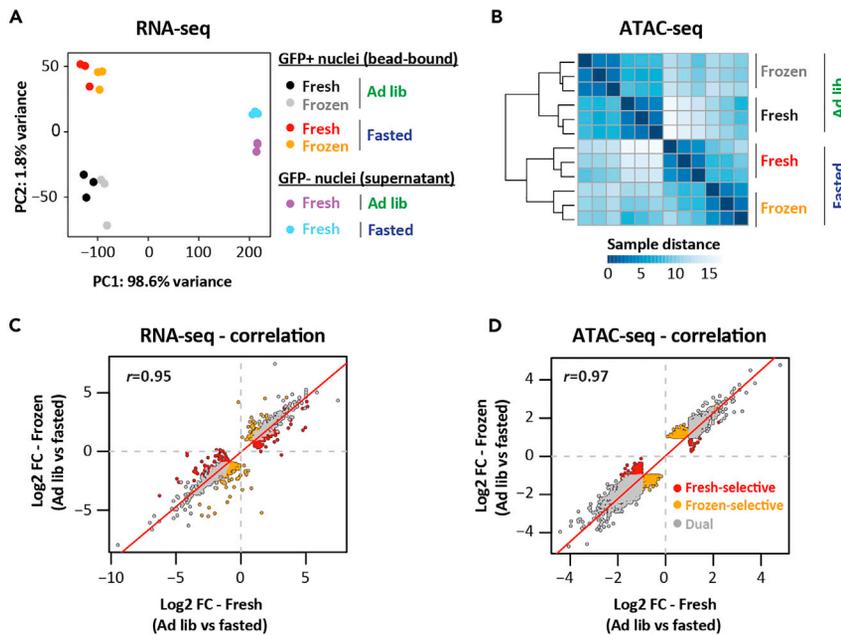


Figure 6. Performance of bead-bound nuclei isolated from fresh and frozen livers in RNA- and ATAC-seq assays
 (A) Principal component (PC) analyses of the RNA-seq data from GFP+ (bead-bound) nuclei obtained from fresh or frozen livers and GFP- (supernatant) obtained from fresh livers of HEP INTACT mice subjected to ad libitum feeding (Ad lib) or an overnight 16 h fast (fasted) (n=3).
 (B) Clustering of the sample-to-sample distance of the 1000 ATAC-seq sites with highest variance identified in GFP+ nuclei isolated from fresh and frozen livers of HEP INTACT mice subjected to ad libitum feeding (Ad lib) or an overnight 16 h fast (fasted) (n=3).
 (C and D) Correlation of log₂ fold changes (log₂ FC) for all (C) differentially expressed genes or (D) differentially accessible sites identified in GFP+ nuclei isolated from fresh and frozen livers (Ad libitum fed vs fasted, padj<0.01, log₂ FC < -1 or > 1). Red, genes/sites only differentially expressed/accessible in the fresh condition; orange, genes/sites only differentially expressed/accessible in the frozen condition; gray, genes/sites differentially expressed/accessible in both conditions. *r* indicates Pearson's correlation.

compared to healthy livers. This protocol uses a 15 mL douncer and up to 1 g of pulverized liver tissue can be effectively dounced using the appropriate amount of LSB (i.e., 4 mL LSB per 0.5 g tissue). It is very important not to overload the douncer with tissue and therefore advisable to dounce multiple rounds if a whole mouse liver (>1 g) is needed and combine before proceeding with the IP.

Problem 2

Nuclei are of poor quality after the initial isolation (steps 3 and 4)

Potential solution

Excessive homogenization might negatively impact the quality of the nuclei. If the nuclei are of a poor quality after the initial isolation, it might be necessary to reduce the number of strokes during douncing. Alternatively, one can try to reduce the incubation time in LSB incl. 0.35% IGEPAL (step 3f) or reduce the concentration of IGEPAL to less than 0.35%.

Problem 3

Nuclei are aggregating after the initial isolation (step 3 and 4)

Potential solution

Using the suggested protocol, we did not experience nuclei aggregations after the initial douncing procedure. However, it is important to completely resuspend the nuclei after the HSB spin, which require that the suggested spin time of 15 min is not exceeded. During the optimization of this protocol, we found that some alternative nuclei isolation protocols using different buffers (e.g., Nuclei

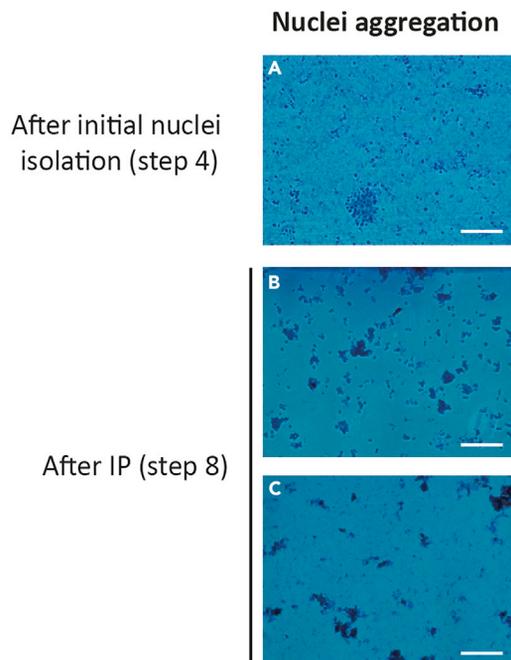


Figure 7. Aggregation of nuclei

Trypan blue-stained nuclei isolated from frozen livers showing nuclei aggregation either due to (A) the use of Nuclei EZ Prep in the initial nuclei isolation or due to (B) the use of an anti-MYC antibody in the IP or (C) vigorously shaking following the IP. Scale bars represent 200 μm .

EZ Prep (NUC101-1KT, Sigma) tended to yield a significant number of aggregating nuclei after the initial isolation (Figure 7A). Therefore, we cannot recommend the use of alternative nuclei isolation buffers unless tested thoroughly.

Problem 4

Nuclei are aggregating after the IP (steps 7 and 8)

Potential solution

Using the suggested protocol, we generally did not find that nuclei aggregation was a major problem after the IP. However, during the optimization of the protocol, we found that some preparations yielded nuclei that clumped heavily together (Figure 7B), which seemed to be highly dependent on the antibody used and the stress posed to the nuclei during the IP. If single nuclei preparations are a strict requirement, it is possible to increase the fraction of these by using a 20 μm filter before or during the IP, but this will result in significant loss of material. It is also possible to add 0.05%–1% BSA to the buffers but note that this should not be done for preparations intended for protein analyses.

Problem 5

Low yield of bead-bound nuclei (steps 7 and 8)

Potential solution

The yield varies greatly depending on the cell type being recovered and the antibody used for the IP and alternative antibodies can be tested to potentially increase the yield. In the original INTACT protocol (Mo et al., 2015), it was suggested that the yield can be increased by performing repeated cycles (5–7 times) of placing the bead-bound nuclei on the magnet stand and then completely resuspending these by inversion (in step 7g). However, at least for hepatocyte nuclei isolated from the liver, we experienced that this led to dramatic aggregation of the hepatocyte nuclei (Figure 7C), making it difficult to use these nuclei for downstream approaches.

Problem 6

Suboptimal ATAC-seq trace after PCR amplification of transposed DNA (steps 13–16)

Potential solution

If only a small fraction of DNA is NFR fragments after PCR amplification (see [Figure 4B](#)), more material will have to be used for the size fractionation to obtain an adequate amount of NFR-associated DNA fragments for sequencing. Adjusting the conditions of the transposition reaction (e.g., the nuclei-to-enzyme ratio or the reaction time) and/or the number of PCR cycles might be able to remedy this problem.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Søren Fisker Schmidt, sfs@bmb.sdu.dk.

Materials availability

This study did not generate new unique reagents.

Data and code availability

The accession codes for the INTACT ATAC-seq and RNA-seq data reported in this paper are GEO: GSE181937 and GEO: GSE181940.

ACKNOWLEDGMENTS

We thank colleagues for input to the protocol and acknowledge the team of technicians at IDC for assistance with mouse work. The graphical abstract was created with [BioRender.com](#). This work was financially supported by grants from the Danish National Research Foundation (DNRF grant No. 141 to the Center for Functional Genomics and Tissue Plasticity [ATLAS]), the Novo Nordisk Foundation (NNFOC150019050) to S.F.S., and the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) through the Collaborative Research Center (CRC) 1118, project number 236360313 to S.H.. A.L. is supported by fellowships from the Novo Nordisk Foundation (NNF16OC0020742), the Danish Independent Research Council | Medical Sciences (DFF-6110-00265), an EMBO Long-Term Fellowship (ALTF 897–2015), and a DZD grant NEXT.

AUTHOR CONTRIBUTIONS

Conceptualization, A.L. and S.F.S.; methodology, A.L. and S.F.S.; formal analyses, A.L. and S.F.S.; investigation, A.L. and S.F.S.; resources, A.L., S.H., and S.F.S.; writing – original draft, A.L. and S.F.S.; writing – review & editing, A.L., S.H., and S.F.S.; visualization, A.L.; project administration, A.L., S.H., and S.F.S.; funding acquisition, A.L., S.H., and S.F.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Buenrostro, J.D., Wu, B., Chang, H.Y., and Greenleaf, W.J. (2015). ATAC-seq: A method for assaying chromatin accessibility genome-wide. *Curr. Protoc. Mol. Biol.* 109, 21 29 21–29.
- Clausen, B.E., Burkhardt, C., Reith, W., Renkawitz, R., and Forster, I. (1999). Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res.* 8, 265–277.
- Corces, M.R., Greenleaf, W.J., and Chang, H.Y. (2019). Isolation of Nuclei from Frozen Tissue for ATAC-Seq and Other Epigenomic Assays (Protocolsio).
- Corces, M.R., Trevino, A.E., Hamilton, E.G., Greenside, P.G., Sinnott-Armstrong, N.A., Vesuna, S., Satpathy, A.T., Rubin, A.J., Montine, K.S., Wu, B., et al. (2017). An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues. *Nat. Methods* 14, 959–962.
- Deal, R.B., and Henikoff, S. (2010). A simple method for gene expression and chromatin profiling of individual cell types within a tissue. *Dev. Cell* 18, 1030–1040.
- Liang, N., Fan, R., Goni, S., and Treuter, E. (2019). preparation of frozen liver tissues for integrated omics analysis. *Methods Mol. Biol.* 1951, 167–178.
- Loft, A., Alfaro, A.J., Schmidt, S.F., Pedersen, F.B., Terkelsen, M.K., Puglia, M., Chow, K.K., Feuchtinger, A., Troullinaki, M., Maida, A., et al. (2021). Liver-fibrosis-activated transcriptional networks govern hepatocyte reprogramming and intra-hepatic communication. *Cell Metab.* 33, 1685–1700.e9.
- Mo, A., Mukamel, E.A., Davis, F.P., Luo, C., Henry, G.L., Picard, S., Urich, M.A., Nery, J.R., Sejnowski, T.J., Lister, R., et al. (2015). Epigenomic signatures of neuronal diversity in the mammalian brain. *Neuron* 86, 1369–1384.
- Postic, C., Shiota, M., Niswender, K.D., Jetton, T.L., Chen, Y., Moates, J.M., Shelton, K.D., Lindner, J., Cherrington, A.D., and Magnuson, M.A. (1999). Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. *J. Biol. Chem.* 274, 305–315.