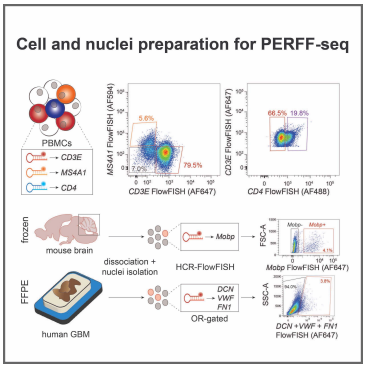

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Protocol status: Working
We use this protocol and it's working

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1. PERFF-seq: Cell and Nuclei Preparation

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ABSTRACT

This protocol can be used for preparation of Cells and Fresh-Frozen and Formalin-Fixed Paraffin-Embedded (FFPE) Tissue Derived Nuclei. This protocol should be followed by "PERFF-seq: HCR-FlowFISH and Polymer Disassembly."

PROTOCOL REFERENCES

1. Reilly, S. K. et al. Direct characterization of cis-regulatory elements and functional dissection of complex genetic associations using HCR-FlowFISH. Nat. Genet. 53, 1166–1176 (2021).
2. HCR™ RNA flow cytometry protocol for mammalian cells in suspension, Molecular Instruments.
3. Tissue Fixation & Dissociation for Chromium Fixed RNA Profiling. 10x Genomics
4. PERFF-seq: HCR Flow-FISH and Polymer Disassembly Protocol

GUIDELINES

- Maintain an RNAse free environment when preparing buffers and throughout the protocol by spraying bench with RNase*Zap*™ and using molecular grade reagents when possible.
- To increase cell recovery, opt for a swinging bucket rotor when pelleting and leave a few uL of liquid behind when aspirating buffers.
- When possible, use low-binding plasticware.

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PROTOCOL integer ID: 94651

Keywords: PERFF-seq, Flow-FISH, Fixation, FFPE, Fresh-Frozen Tissue, Tissue Dissociation

MATERIALS

FOR FRESH NUCLEI PREP

1. Dissociation Solution *(Liberase TL (Lot No: 5401020001) + RPMI (with L-glutamine))*
2. gentleMACS Octo Dissociator with Heaters
3. gentleMACS C Tubes
4. Miltenyi Pre-Separation Filters (30um)
5. DAPI (1mg/mL)
6. Fixation Buffer *(4% Paraformaldehyde + 1x PBS + 0.1% Tween 20)*
7. 1x PBS
8. Permeabilization Buffer *(Freshly prepared 70% Ethanol)*

FOR FFPE NUCLEI PREP

1. FFPE Block
2. 100% Ethanol
3. CitriSolv
4. 1x PBS
5. Miltenyi Pre-Separation Filters (30um)
6. DAPI (1mg/mL)
7. Fixation Buffer *(4% Paraformaldehyde + 1x PBS + 0.1% Tween 20)*
8. PBS-T *(1x PBS + 0.1% Tween 20)*
9. Permeabilization Buffer *(Freshly prepared 70% Ethanol)*

FOR CELL PREP

1. Staining Buffer *(1x PBS + 1% BSA)*
2. PBS-T *(1x PBS + 0.1% Tween 20)*
3. Fixation Buffer *(4% Paraformaldehyde + 1x PBS + 0.1% Tween 20)*
4. Permeabilization Buffer *(Freshly prepared 70% Ethanol)*

GENERAL REAGENTS & INSTRUMENTS

1. Storage Buffer *(Water + 1x Quenching Buffer + 0.1 Enhancer)*
2. Protector RNAse Inhibitor *(Sigma-Aldrich Lot No: 3335399001)*

SAFETY WARNINGS

Use formaldehyde with caution as it is a hazardous material.

BEFORE START INSTRUCTIONS

Calculate the starting cell count and volume of probes and hairpins needed for your experiment by putting these factors into account:

- Proportion of the specific population of interest within the total cell/nuclei population.
- Final cell count should be >50,000.
  i.e specific population of interest to sort
- Approximately 50% of starting cells/nuclei are lost during the wash steps of this protocol.
- Unstained controls and single color controls (if multiplexing)

$$Starting\ Cell\ Count = \frac{Final\ Cell\ Count}{Percentage\ of\ subpopulation\ of\ interest} * 200$$

Nuclei Preparation from Fresh Frozen Tissue

1 FIXATION AND PERMEABILIZATION

1.1 Weight tissue to determine the volume of fixation buffer needed.
- 2 mL fixation buffer per 25 mg tissue is required.

1.2 Place tissue on a pre-chilled glass petri dish maintained On ice and using a blade, mince tissue finely (enable passing through a 1mL wide-bore pipette tip)

1.3 Using a wide bore 1mL pipette, add the required fixation buffer depending on the amount of tissue.

1.4 Incubate at room temperature for 2-3 hours with intermittent shaking. 25 °C

1.5 Centrifuge at 850xg for 5 minutes.
Gently remove supernatant without disturbing the pellet.
Add 2mL PBS per 25mg tissue to wash tissue.
Centrifuge at 850xg for 5 minutes.

1.6 Add 2mL permeabilization buffer per 25mg tissue and incubate overnight at 4 °C to permeabilize.

1.7 Centrifuge at 850xg for 5 minutes.
Remove supernatant without disturbing the pellet.
Add 2mL PBS per 25mg tissue to wash tissue.
Centrifuge at 850xg for 5 minutes.

2 TISSUE DISSOCIATION

2.1 Make the dissociation solution as follows:

- Reconstitute 5mg Liberase (5401020001) with 1mL sterile water. Mix until fully dissolved and make single use aliquots for future use in -20 °C .

	A	B
	Contents	For ≤100mg tissue
	RPMI (w L-glutamine)	1840uL
	Liberase	160uL

Keep dissociation solution On ice until ready to proceed.

Warm the solution for 10 minutes at 37 °C .

2.2 Add 2mL per ≤100mg pre-warmed dissociation solution to the sample.
Re-suspend and transfer 2mL of sample into each C tube.
Run the following program on the gentleMACS Octo dissociator with heaters ON:

	A	B
	Step	Program
	1	Temp ON
	2	Spin 50 rpm, 40’ 0 “
	3	Spin 1000rpm, 30”
	4	Spin -1000rpm, 30”
	5	end

2.3 Ensure all tissue pieces are in suspension when flipping C tubes over for dissociation.
Once the program is done, ensure all tissue is dissociated before proceeding.

- If there are large chunks of tissue that are undissociated, run a 50 rpm spin only for longer until chunks are dissociated.

2.4 Perform a quick pulse centrifuge (300xg) to collect all cells into the bottom of the tube.

2.5 Re-suspend and pass the dissociated tissue through a 30um filter to remove debris and tissue chunks.

2.6 Perform an additional wash by adding 2mL of PBS into tubes and passing through the same filter.

2.7 Count nuclei on countess or hemocytometer and record nuclei count.

- Expect > 4 million nuclei per 25mg of tissue.

2.8 Stain with 1uL of DAPI (1mg/mL) per 5 million cells and sort DAPI+ nuclei.

2.9 Proceed immediately with the PERFF-seq: HCR Flow-FISH and Polymer Disassembly Protocol.

Stopping point:
Alternatively, samples can be resuspended in storage buffer (1mL per 5 million, see Materials section or 10XG protocol for more details).
Nuclei can be stored for up to 1 week at 4 °C .

Nuclei Preparation from Formalin Fixed Paraffin Embedded (FFPE) Tissue …

3 **ISOLATION**

3.1 Obtain 2 to 6 FFPE curls (Ideally 50uM to 70uM of thickness).
Total number and thickness of each curls will change based on how much tissue is embedded and desired number of nuclei.
Curls can be stored in a 1.5mL tube at 4 °C for long term storage.

3.2 For each sample, pre-cool 2 NIC+ cartridges (S2 Genomics) be placing them in the fridge.
Prepare the following reagents in 15mL tubes (per sample).
- 5mL Ethanol (100%)
- 5mL CitriSolv (solvent)
- 5mL of PBS (-Ca/Mg)
- Empty 50mL tube for waste

3.3 Place reagent lines from S200+ Singulator Prototype into appropriate 15mL conicals.
a. Place Waste line into empty 50mL tube.
b. Place Solvent line into CitriSolv.
c. Place Ethanol line into Ethanol.
d. Place Buffer line into PBS.
Add curls to NIC+ cartridge.
Make sure the curls are on the bottom of the cartridge chamber.

3.4 Turn on Singulator and tablet.

Insert cartridge and start the FFPE extraction protocol.

3.5 The protocol (~50 minutes) will run deparaffination with CitriSolv, rehydration with Ethanol, and washes with PBS.
a. 15 minute CitriSolv incubation.
b. 7.5 minute CitriSolv incubation.
c. 7.5 minute CitriSolv incubation.
d. 1 minute 100% Ethanol incubation.
e. 1 minute 100% Ethanol incubation.
f. 1 minute 70% Ethanol incubation.
g. 1 minute 50% Ethanol incubation.
h. 1 minute 30% Ethanol incubation.
i. 3x PBS washes.
During the run, pre-cool Singulator 200 for subsequent nuclei extraction from tissue.
Upon completion of the protocol, remove the cartridge from the machine.

3.6 Using a P1000 pipette, remove the sample (will appear as small tissue pieces) and transfer into a 5mL conical tube On ice (Pipette gently, trying to not break further pieces of tissue during transfer).
Centrifuge at 1000g for 3 minutes at 4 °C.
Remove the supernatant and resuspend in 500 µL of Nuclei Isolation Reagent (NIR).
Add 12.5 µL of Protector RNase Inhibitor (1U/uL final).
Then transfer 400 µL of NIR+ tissue to a new pre-cooled NIC+ cartridge.
Select FFPE Nuclei Isolation protocol.
Place cartridge in Singulator 200 and start protocol.
While Singulator is running (about 8 minutes), add 25uL of Protector RNase Inhibitor to 1mL of Nuclei Storage Reagent (NSR) (1U/uL final).
Upon completion of the run, remove the cartridge and pierce the foil to collect the isolated nuclei.

3.7 Transfer nuclei suspension into 5mL tubes On ice.
Centrifuge at 500xg for 5 minutes at 4 °C.
Remove gently supernatant and resuspend in 1mL of NSR + Protector RNase Inhibitor (1U/uL final).

3.8 Filter nuclei suspension with Miltenyi Biotec Pre-Separation Filters (30um).
Count nuclei using DAPI.
Trypan can be used when counting with brightfield, but DAPI will yield more accurate counts.

4 FIXATION AND PERMEABILIZATION

4.1 Spin nuclei at 850xg for 5 minutes at 4 °C and gently remove supernatant.

4.2 Resuspend and add fixation buffer to fix cells at 1M/mL concentration.

4.3 Incubate at room temperature for one hour or 4 °C overnight.

4.4 After incubating, centrifuge at 850xg for 5 minutes and aspirate supernatant.

4.5 Resuspend in 1x PBS + 0.5% BSA + 0.2U/uL Protector RNase Inhibitor (final) at 1M/1mL concentration On ice .

4.6 Centrifuge at 850xg for 5 minutes at 4 °C and aspirate supernatant.

4.7 Resuspend in 1x PBS + 0.5% BSA + 0.2U/uL Protector RNase Inhibitor (final) at 1M/1mL concentration On ice .

4.8 Filter nuclei suspension through a 35um FACS tube cap filter (blue) or Miltenyi Biotec Pre-Separation Filters (30um).

4.9 Stain with 1uL of DAPI (1mg/mL) per 5 million cells and sort DAPI+ nuclei.
DAPI+ nuclei can be sorted (suggested nozzle of 100um) into 150uL of 1x PBS + 0.5% BSA + 0.2U/uL Protector RNase Inhibitor (final).
Use 1.5mL Lobind Eppendorf as collection tube.

4.10 Centrifuge sorted nuclei at 850xg for 5 minutes at 4 °C and gently remove supernatant.

4.11 Add ice cold permeabilization buffer to nuclei at 1M/mL concentration.
Incubate at 4 °C overnight.

4.12 Remove samples from 4 °C and centrifuge at 850xg for 5 minutes.

4.13 Add 1M/mL of PBST and **count nuclei** to ensure minimal cell loss post fixation and permeabilization.
Nuclei can be split into 1 million - 5 million nuclei aliquots for HCR Flow-FISH experiments.
Centrifuge at 850xg for 5 minutes.
Repeat wash with 500uL of PBST.

4.14 Proceed immediately with the PERFF-seq: HCR Flow-FISH and Polymer Disassembly Protocol.
Stopping point:
Alternatively, samples can be resuspended in storage buffer (1mL per 5 million, see Materials section or 10XG protocol for more details).
Nuclei can be stored for up to 1 week at 4 °C.

Preparation of Peripheral Blood Mononuclear Cells (PBMCs) & Cell Lines

5 ANTIBODY STAINING (Optional): For Use in conjunction with HCR FlowFISH

Note

For enrichment applications based on HCR FlowFISH and surface antibody staining, we recommend performing surface staining before fixing and permeabilizing cells.

- We note that surface antibodies conjugated to synthetic dyes result in the most robust signal when used in conjunction with our HCR FlowFISH protocol.
- For downstream dead cell exclusion, a fixable viability dye can be used prior to fixation and permeabilization.

5.1 Re-suspend cells with 45uL of staining buffer. Add 5uL of Fc block to cells and incubate at 4 °C for 15 minutes.

5.2 Make antibody cocktail based on manufacturer recommendations and re-suspend cells.
Incubate in dark at 4 °C for 20 minutes.
Wash 2 times with staining buffer to remove unbound antibodies.
If using a fixable viability dye, staining can be done here.

6 FIXATION AND PERMEABILIZATION

6.1 Add fixation buffer to fix cells at a 1M/mL concentration.

- For example, re-suspend 50M cells in 50mL of fixation solution

6.2 Incubate at room temperature for one hour or 4 °C overnight.
After incubating, centrifuge at 850xg for 5 minutes.
Wash 2 times with PBS-T at 1M/mL concentration.

6.3 Add ice cold permeabilization buffer to cells at 1M/mL concentration.

Incubate at 4 °C overnight.

6.4 Remove samples from 4 °C and centrifuge at 850xg for 5 minutes.

6.5 Add 1M/mL of PBST and **count cells** to ensure minimal cell loss post fixation and permeabilization.
Cells can be split into 1 million - 5 million cell aliquots for HCR Flow-FISH experiments.
Centrifuge at 850xg for 5 minutes.
Repeat wash with 500uL of PBST.

6.6 Proceed immediately with the PERFF-seq: HCR Flow-FISH and Polymer Disassembly Protocol.

Stopping point:

Alternatively, samples can be resuspended in storage buffer (1mL per 5 million, see Materials section or 10XG protocol for more details).

Cells can be stored for up to 1 week at 4 °C.