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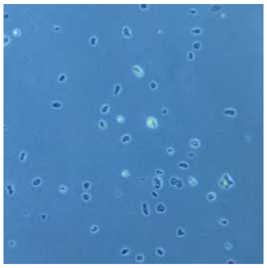
# snPATHO-seq



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Tony Wang[1], Michael Roach[2], Jasmine Plummer[3], Luciano G Martelotto[2]

[1]Garvan Medical Institute; [2]Adelaide Centre for Epigenetics, University of Adelaide; [3]University of Adelaide

Jasmine Plummer: Center for Spatial OMICs, St Jude Children's Research Hospital;

Human Cell Atlas Metho... Single Cell Core, Harvar... 1 more workspace

Michael Roach
Flinders Health and Medical Research Institute, Flinders Uni...

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

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## Abstract

Formalin-fixed paraffin-embedded (FFPE) samples are valuable but under-utilised in single-cell omics research due to their low DNA and RNA quality. Leveraging recent single-cell genomic technology advances, we introduce snPATHO-seq: a versatile method to derive high-quality single-nucleus transcriptomic data from FFPE samples.

# Materials

**Reagents and consumables**

- Ethanol
- Xylene
- Nuclease Free water
- 1x Phosphate Buffer Saline (PBS, Ca2+ and Mg2+ free)
- Liberase TM or TH (Roche)
- Collagenase D or P (Roche)
- Hyaluronidase (CAS 37326-33-3, Calbiochem)
- RPMI1640 (Gibco)
- EZ Lysis Buffer (Sigma)
- 10% BSA (MACS® BSA Stock Solution, Miltenyi)
- Glycerol 50%
- RNAse Inhibitor (RiboLock from Thermo or RNA Protector from Roche)
- (Optional) 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, ThermoFisher)
- 40 um and 70 um pluriStrainer filters (pluriSelect) (MACS SmartStrainers are also possible)
- 25 G needle

**Equipment**

- Thermomixer with adjustable shaking (Eppendorf)
- Swinging bucket refrigerated centrifuge

# Troubleshooting

## Paraffin removal

1 Cut 1 or 2 approximately 25 µm-thick sections (punches are also possible) and transfer to a 1.5 mL Eppendorf tube.

> **Note**
>
> Store dry at 4°C if not used immediately. To keep it dry store in a container with silica beads. You can use the cylinder with silica beads that comes with 10x Genomics chips.

2 Add 1 mL Xylene and incubate for 00:10:00 at Room temperature or 50-55 °C 10m

> **Note**
>
> Optional: heat at 50-55°C for 10 mins for the first xylene wash. This has proven to be very helpful in removing the paraffin more efficiently, in particular with fatty tissue.

3 Carefully remove Xylene without disturbing the sample.

4 Repeat steps 2 and 3 two more times: 20m

Add 1 mL Xylene , incubate 00:10:00 at Room temperature , remove xylene

Add 1 mL Xylene , incubate 00:10:00 at Room temperature , remove xylene

## Rehydration

5 1m

> **Note**
>
> **OVERVIEW**: Wash sections with sequential ethanol immersions: 2x 100%, 1x 70%, 1x 50%, 1x 30%). Lastly, wash with RPMI1640.

Add 1 mL 100% ethanol and incubate for 00:01:00 at Room temperature , then carefully remove ethanol without disturbing the sections/punches.

6 Repeat step 5: add 1 mL 100% ethanol , incubate for 00:01:00 at Room temperature , remove ethanol wash 1m

7 Add 1 mL 70% ethanol , incubate for 00:01:00 at Room temperature , remove ethanol wash 1m

8 Add 1 mL 50% ethanol , incubate for 00:01:00 at Room temperature , remove ethanol wash 1m

9 Add 1 mL 30% ethanol , incubate for 00:01:00 at Room temperature , remove ethanol wash 1m

10 Add 1 mL RPMI1640 , incubate for 00:01:00 at Room temperature , carefully remove RPMI1640 wash 1m

## Tissue dissociation

11 Prepare 1 mL Dissociation Solution :

- 1 mL RPMI1640
- [M] 0.25-1 mg/mL Liberase TM(*)
- [M] 0.25-1 mg/mL 10x Collagenase D(**)
- [M] 0.25-1 mg/mL Hyaluronidase
- [M] 1 U/uL RNAse Inhibitor

Add this 1 mL Dissociation Solution to the sections/punches.

Note

(*) Liberase TH is very good enzyme too and can be used alone at 1-2.5 mg/mL.
(**) Collagenase P (Roche) works fine as well.

**IMPORTANT:** I strongly recommend testing what concentration of enzymes works best for your tissue of interest. In our experience, Liberase TH alone at 1 mg/mL or the trio at 1 mg/mL Liberase TM + 1 mg/mL Collagenase D + 0.5 mg/mL Hyaluronidase works well with most of the samples we tested.

- **Pro tip**: Add 200 µL of the enzymatic cocktail and mince using a pestle for at least 10 strokes, then complete to 1 mL with the rest of the cocktail mix.
- **Optional Pro tip**: Before digestion, add 100 µL of digestion mix and homogenize the sample using a douncer/pestle by stroking 10-20 times. This helps in the digestion step. Then top up with the rest of the digestion mix.

12 Digest tissue for 45-60 mins(*) at 37°C in a Thermomixer at 800 RPM.

800 rpm, 37°C, 00:45:00 in Thermomixer

45m

Note

(*) Some blocks require longer digestion time. Inspect visually and help dissociation by pipetting up and down with a P1000 pipette.

**IMPORTANT**: Dissociation does not need to be complete; the objective here is to loosen up the material to facilitate the nuclei release. Dissociation completeness varies from block to block. Tissue does not need to be fully digested.

## Lysing the cells

13

5m

Note

**OVERVIEW**: Wash with lysis buffer. Resuspend and homogenize in small volume of lysis buffer. Add rest of lysis buffer and homogenise several times.

Add 400 µL Ez Lysis Buffer to the sample and mix by inverting 5 times, then centrifuge 850 rcf, 4°C, 00:05:00

14 Prepare 2 mL Lysis Solution as follows:

- 2 mL Ez Lysis buffer
- [M] 2 % (v/v) BSA
- [M] 1 U/uL RNAse Inhibitor

15 Remove supernatant and add 250 µL Lysis Solution (from step 14)

16 Homogenize the sample using a douncer/pestle by stroking 10-20 times (or as needed).

17 Add a further 750 µL Lysis Solution (from step 14)

18 Homogenize by pipetting using a P1000 pipette (10 times), then incubate On ice for 00:05:00 5m

19 Repeat step 18: pipette 10 times and incubate On ice for 00:05:00 5m

20 **Optional but very useful when possible**: If the dissociation and disaggregation look almost complete (i.e. only very small chunks of undigested tissue or fat are visible to the naked eye) gently pass the sample through a 25G needle for 20 times (avoid foaming). It is essential to ensure that no large chunks remain *before* passing though needle. If large chunks or fat remain the needle will definitely block, so just skip this step. This optional step will increase the nuclei release.

## Cleaning the nuclei

21

> Note
>
> **OVERVIEW**: Filter (large pore size). Wash with lysis solution. Wash with PBS. Filter (small pore size).

Prepare 5 mL Wash Solution as follows:

- 5 mL 0.5x PBS
- [M] 0.02 % volume BSA

22 Pass the sample through a 70 µm PluriStrainer filter to remove large chunks of undigested tissue.

Note

**Do not use a FLOWMI cell strainer!**

23 Centrifuge the flow-through 850 rcf, 4°C, 00:05:00 5m

24 Remove supernatant and resuspend with 800 µL Lysis solution (from step 14)

25 Centrifuge 850 rcf, 4°C, 00:05:00 5m

26 Remove supernatant and resuspend in 500-1000 µL Wash solution (from step 21)

Note

Resuspension volume can vary depending on the pellet size.

27 Centrifuge 850 rcf, 4°C, 00:05:00 5m

28 Repeat wash steps 26 and 27: remove supernatant, resuspend in 500-1000 µL Wash solution (from step 21) and centrifuge 850 rcf, 4°C, 00:05:00 5m

29
- Remove supernatant and resuspend in 500-1000 µL Wash solution (from step 21)
- Pass sample through a 40 µm PluriStrainer filter

Note

**Do not use a FLOWMI cell strainer!**

## Using the nuclei

15m

30 Count using Luna-FX7 or similar based on dual-fluorescence such as AO/PI.

31

Note

Cycling conditions for Index PCR might need to be optimized per sample to obtain a final library that falls within ~50-200 nM. For nuclei derived from FFPE blocks, we typically use 1-2 additional cycles during indexing to start with. If the library does not reach the recommended range but the Bionalyzer/Fragment Analyzer/Tapestation traces look as expected (single peak at ~265 bp), then do not add additional cycles. If you see signs of under/over amplification in the traces, then adjust cycling accordingly.

STEP CASE

### Steps for immediate usage

1 step

32 Rest on wet ice for immediate FACS cytometry analysis/sorting, or proceed to the Chromium X run using Chromium Fix RNA Profiling (10x Genomics) following the user guide for singleplexed or multiplexed samples accordingly.