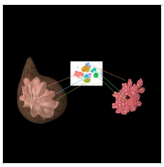

In devel.

Single Nuclei RNA Sequencing of Breast Adipose Tissue (10x Nuclei-Seq)

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Human Cell Atlas Method Development Community

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ABSTRACT

Breast cancer originates in the mammary gland epithelium, however growing evidence demonstrates that the diverse array of stromal tissues influence the behavior of breast epithelium and has key roles in the pathogenesis of breast cancer. Despite increased recognition and research, the heterogeneity of the breast stroma (endothelium, fibroblasts, and adipocytes) poses challenges in elucidating which stromal populations are responsible for the complex interactions of the breast microenvironment. To this end, we have employed single cell RNA sequencing (scRNAseq) of the stromal populations within the breast. However adipose tissue, due to its delicate and lipid filled nature is not amenable to these methods of interrogation. To overcome this obstacle, we have developed a method for isolation of adipose nuclei for 10x sequencing. Together, we will use these approaches to investigate the heterogeneity of stroma and adipocytes, and determine the interactions of the breast microenvironment at single-cell resolution.

TAGS

Single Nuclei RNA-Seq

single-nuclei RNA-Seq

Show tags

PROTOCOL STATUS

In development

We are still developing and optimizing this protocol

GUIDELINES

1. Use of this protocol for other tissues.
- It is important to note that additional optimization is required for different tissues.
-

- In particular it is important to optimize the concentration of lysing detergent (in this case NP-40) for the tissue. Also the amount of BSA in the wash buffer requires optimization.
-

- Centrifugation speeds should be also be optimized. Our experience shows that nuclei after FACS purificaiton are lysed when centrifuged at 500xg. Centrifugation at slower speeds for longer periods are necessary to pool nuclei.
-

1. Quality Control
- QC for nuclei can be difficult by metrics typically used for cells (ie trypan blue marks nuclei, but is used to identify dead cells). The most important is microscopy throughout the nuclei preparation to ensure that nuclei remain of high quality before proceeding with 10xseq
-

1. FACS purification.
- FACS purification of nuclei after tissue preparation is paramount for successful 10x Nuclei Seq.
-

- Lack of FACS purificaiton results in excessive amounts of ambient RNA that results in poor results--low UMI, falsely high

number of cells (Figure 1) and poor indisitnct tSNE clustering (Figure 2)

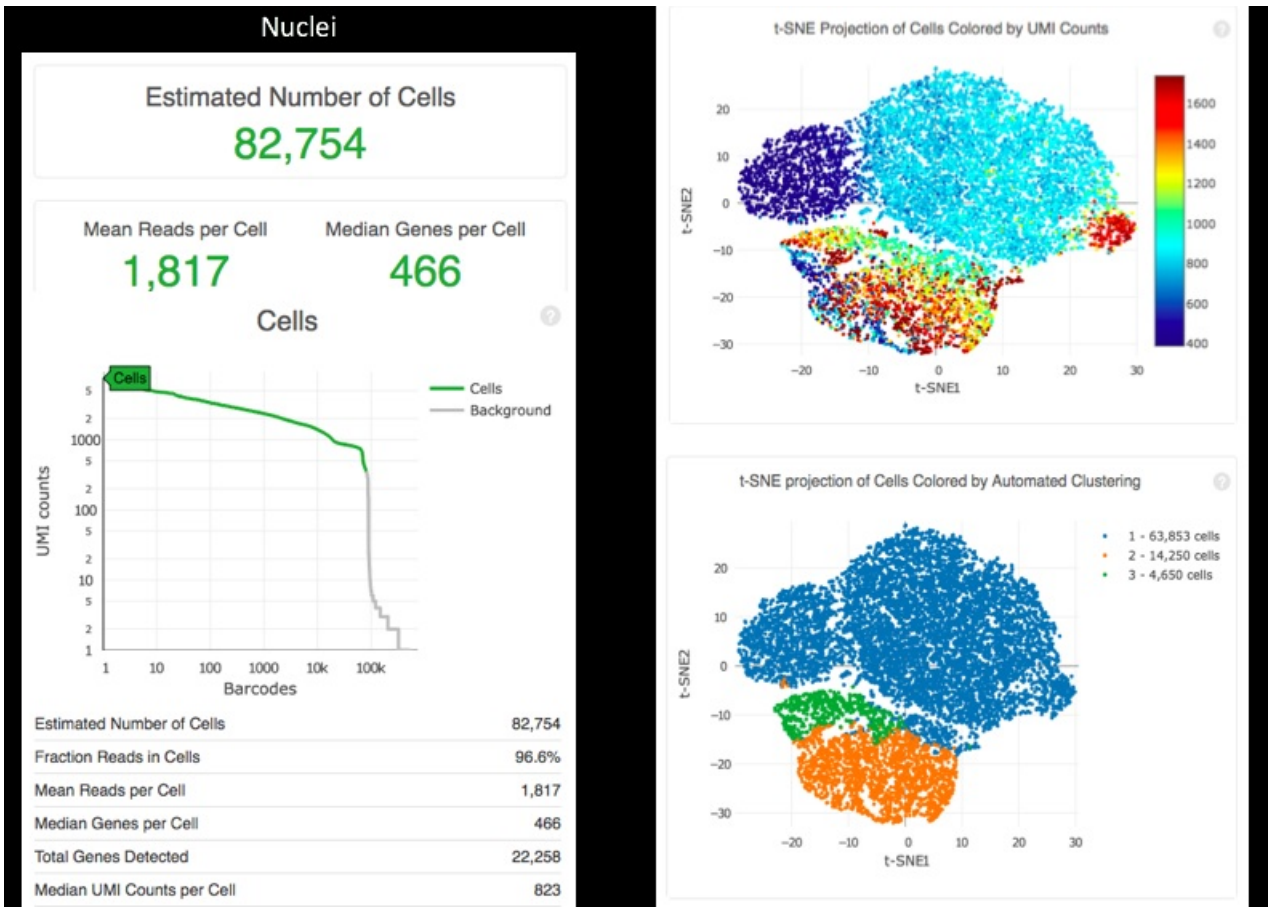


Figure 1--Ambient RNA resulsts in false positive cells: Only 10K nuclei were loaded. But 82K nuclei were detected, also a majority of 'cells' head low UMI and gene counts.

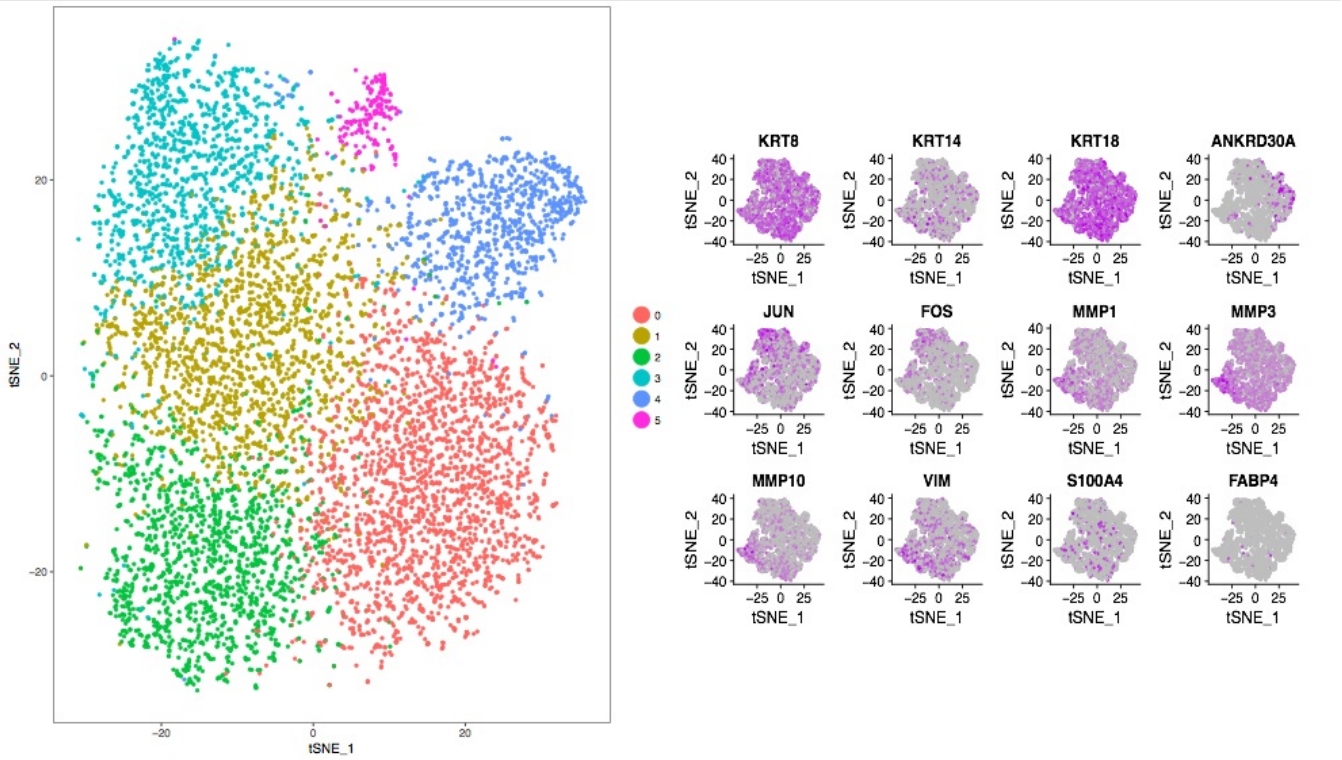


Figure 2--Ambient RNA results in poor clustering: ambient RNA from nuclei that were not FACS purified results in tSNE clustering where marker genes for various cells types are found in all cells.

MATERIALS

NAME	CATALOG #	VENDOR

Prepare Materials

1

Nuclear Isolation Buffer (NIB)

400ul 1M Tris-HCl pH=7.4
80ul 5M NaCl
120ul 1M MgCl2
400ul 10% NP40
39mls sterile H2O

Nuclear Wash Buffer (NWB)

1x PBS
0.5% BSA
0.2U/ul Rnase Inhibitor

REAGENT
1M Tris-HCl pH=7.5
by Invitrogen - Thermo Fisher
Catalog #: 15567-027

REAGENT
5M NaCl
by Ambion
Catalog #: AM9760G

REAGENT
1M MgCl2
by Ambion
Catalog #: AM9530G

REAGENT
Surfact-Amps NP-40
by Thermo Fisher Scientific
Catalog #: 28324

REAGENT
Protector RNase Inhibitor
by Sigma Aldrich
Catalog #: 03335399001

REAGENT
Albumin, Bovine Serum, 10% Aqueous Solution, Nuclease-Free
by Millipore Sigma
Catalog #: 126615-25ML

REAGENT
UltraPure Distilled Water
by Invitrogen - Thermo Fisher
Catalog #: 10977-015

REAGENT
10x PBS
by Thermo Fisher Scientific
Catalog #: AM9624

Prepare Tissue for Dounce Homogenization

2 1. Place homogenizer on ice in preparation for nuclei isolation.
2. Cut away 40g of adipose tissue from epithelium and stroma.
3. Mince 10g of tissue at a time into fine (~2mm x ~2mm) pieces. Tissue is minced in portions to avoid lipolysis of adipocytes that will occur if it is all minced together initially.
4. Place tissue into 15ml dounce homogenizer.

EQUIPMENT
Fisher Scientific K885300-0015
15ml Kimble™ Kontes™ Dounce Tissue Grinders

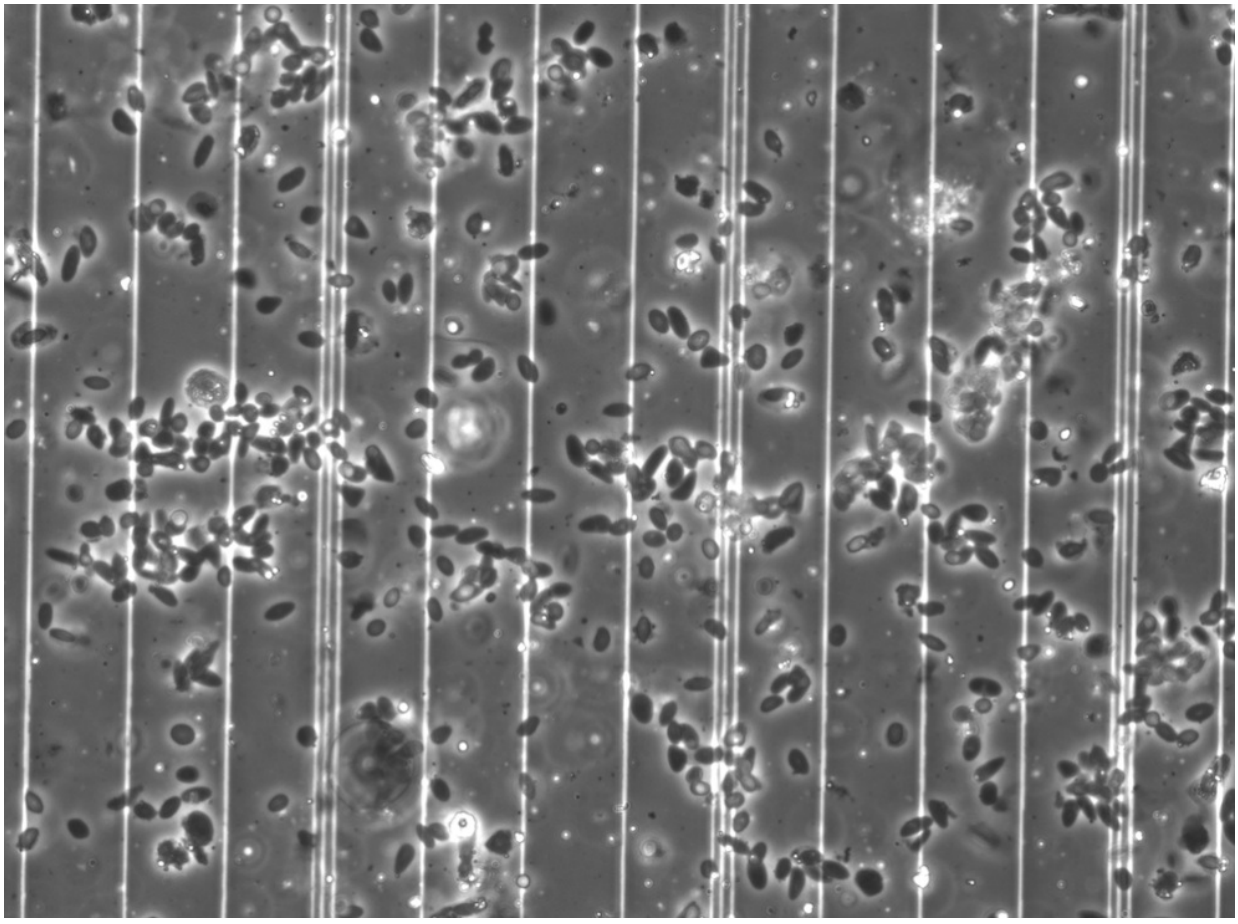
Isolate Nuclei

3 1. Add 10ml of NIB to homogenizer.
2. Slowly push down the tissue with the A piston
3. Run piston through the tissue 10-15, until the piston can smoothly move up and down.
4. Use a 10ml pipette to transfer the fat/nuclei suspension from the dounce homogenizer into a 50ml conical through a 70uM mesh filter. Change filter if it becomes clogged.
5. Repeat steps 2 and 3 three times until all of the tissue has been homogenized in 10g portions.

4 °C Steps should generally be performed on ice as to preserve the integrity of nuclei

EXPECTED RESULT

Nuclei should be inspected throughout the preparation with a bright-field microscope. There will be significant debris before FACS purification of nuclei



Wash and Stain Nuclei

4 1. Centrifuge Nuclei/Tissue suspension at 500xg for 5 mins at 4C.
2. Remove supernatant carefully. There will be a lipid layer supernatant, it is important to remove this layer first, as removing the subnatant first can result in a lipid/nuclei suspension that is difficult to separate.
3. Resuspend nuclei pellet in 1ml of NWB and strain through 35uM filter into a FACS tube.
4. Centrifuge nuclei in NWB at 500xg for 5mins at 4C.
5. Remove supernatant and resuspend nuclei pellet in NWB with 10ug/ml of Hoechst.
6. Nuclei are ready for FACS purification.

EQUIPMENT

Corning 352235

FACS tubes with strainer

4 °C **Centrifugation steps should be perfromed at 4C**

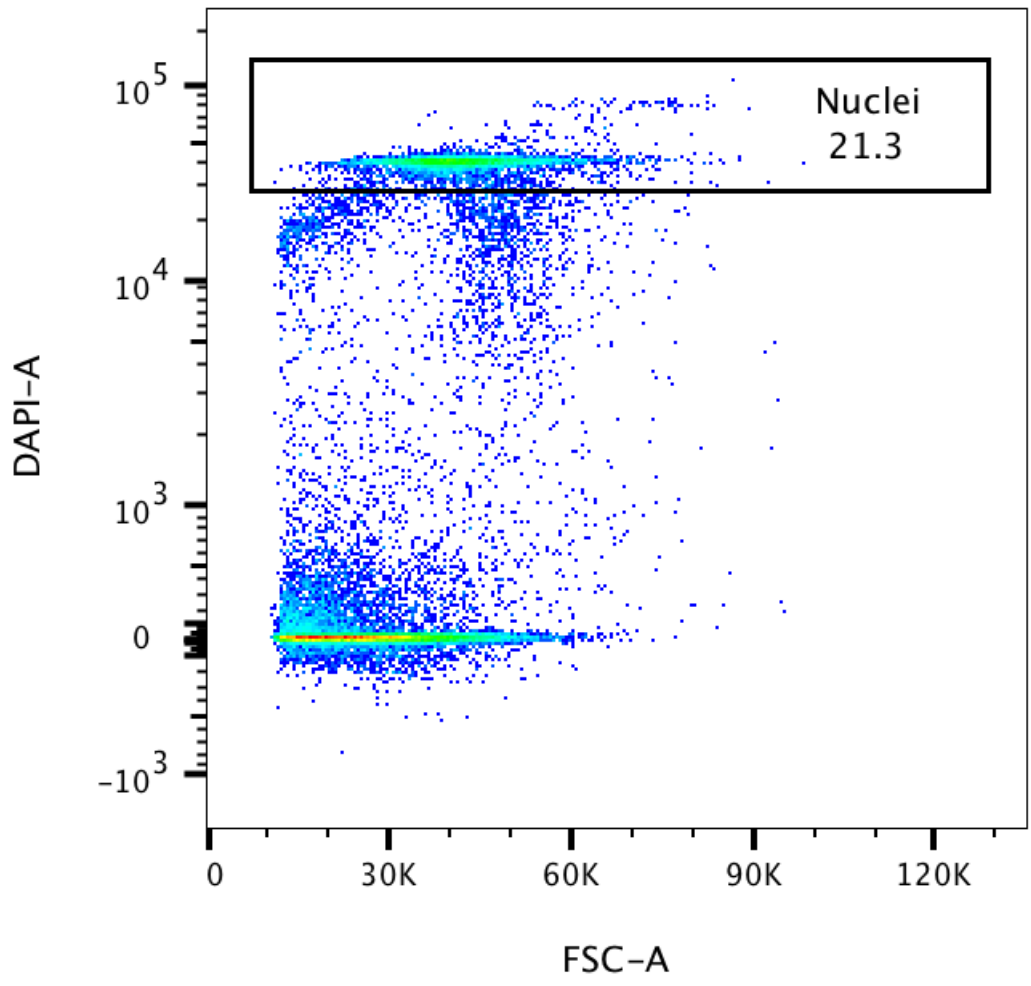
REAGENT
Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution in Water
by Invitrogen - Thermo Fisher
Catalog #: H3570

FACS Purification of Nuclei

5 FACS Purification was performed using a Becton-Dickinson FACS Aria

Here is an example of the expected final gate that will have Hoescht+ Nuclei :

EXPECTED RESULT



Post-FACS Preparation of Nuclei for 10xSeq

6

1. Sort nuclei into FACS tubes in 1ml of NWB
2. Centrifuge nuclei at 200xg for 10mins.
3. Resuspend nuclei in appropriate volume of NWB that will achieve a suspension of ~1000 nuclei/ul.
4. Count nuclei using both Countess and hemacytometer with trypan blue.

- Nuclei counted with trypan blue will be counted as 'dead' by the automated cell counter
-

- There may be a discrepancy between the countess and hemacytometer. Recount if error is >5%.
-

1. Nuclei are ready for loading for 10xSeq. Proceed with manufacturer's instructions.

EQUIPMENT

Thermo Fisher AMQAX1000

Countess II-Automated Cell Counter

EXPECTED RESULT

EXPECTED RESULT