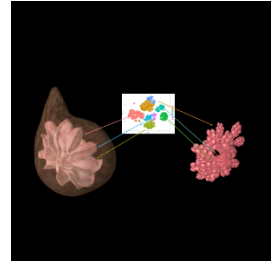


Sep 25, 2018

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

DOI

dx.doi.org/10.17504/protocols.io.tdwei7e



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Human Cell Atlas Metho...

10X Genomics



Kevin Nee

University of California-Irvine, Medical Scientist Training ...

OPEN  ACCESS



DOI: dx.doi.org/10.17504/protocols.io.tdwei7e

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Protocol status: In development

We are still developing and optimizing this protocol

Created: September 10, 2018

Last Modified: September 25, 2018

Protocol Integer ID: 15510

Keywords: snRNAseq, Single Nuclei RNA Sequencing, Breast Adipose, Adipocyte, Adipose

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.



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 purification 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 purification results in excessive amounts of ambient RNA that results in poor results--low UMI, falsely high number of cells (Figure 1) and poor indistinct tSNE clustering (Figure 2)
-

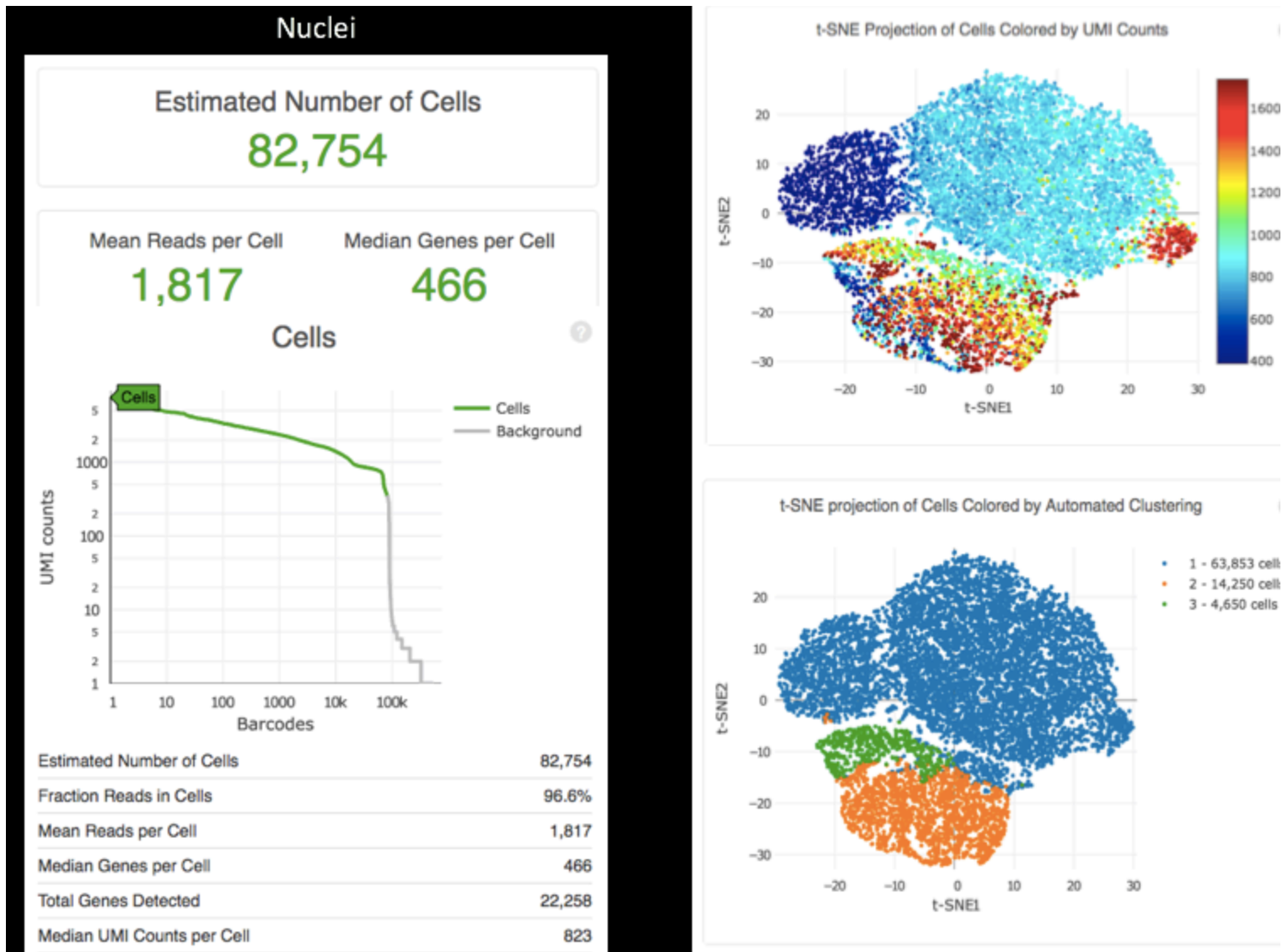


Figure 1--Ambient RNA results in false positive cells: Only 10K nuclei were loaded. But 82K nuclei were detected, also a majority of 'cells' had low UMI and gene counts.

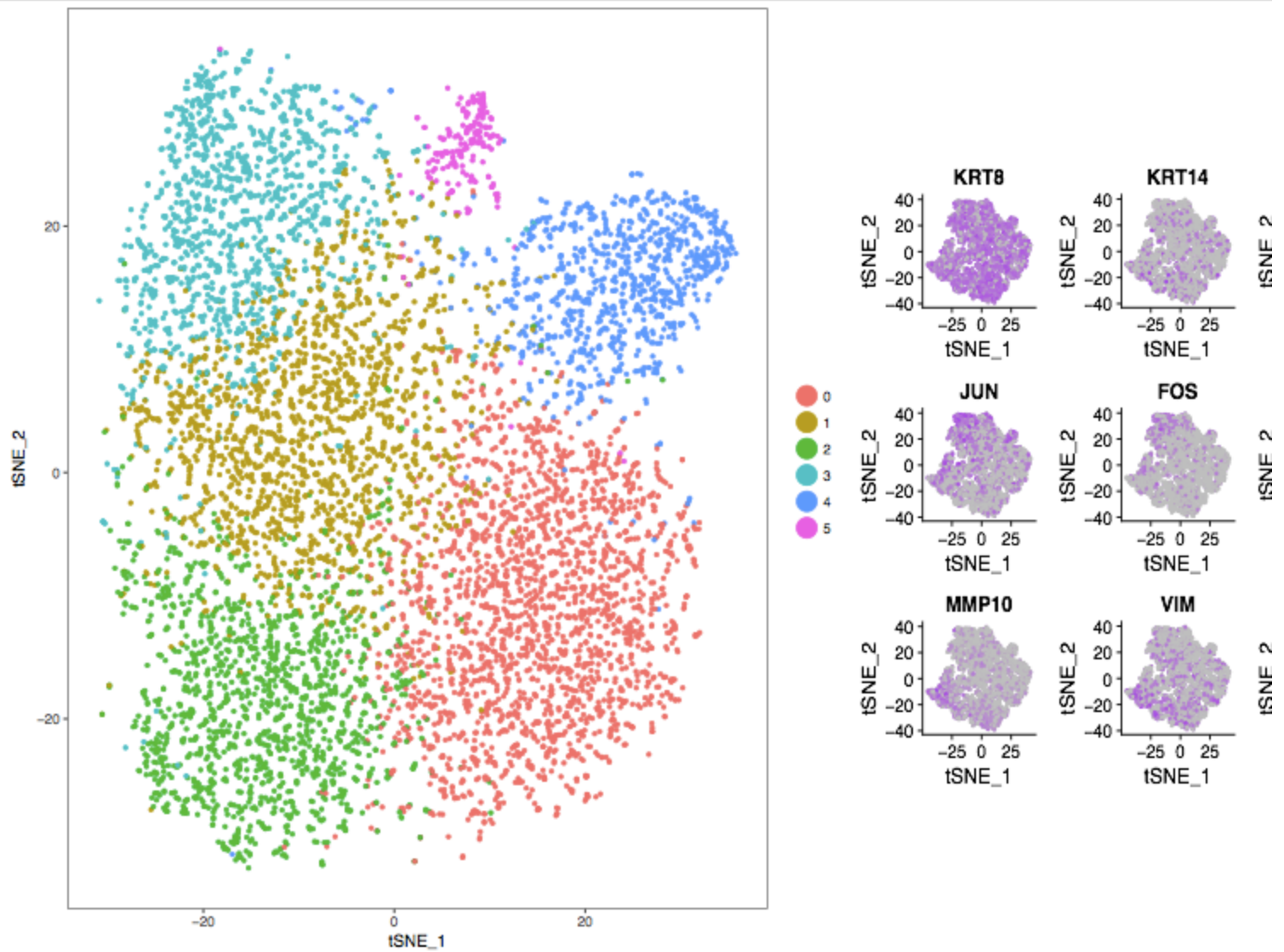


Figure 2--Ambient RNA results in poor clustering: ambient RNA from nuclei that were not FACS purified results in tSNE clustering where marker genes for various cells types are found in all cells.



Materials

MATERIALS

☒ PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific Catalog #AM9625**

☒ 1M MgCl₂ **Ambion Catalog #AM9530G**

☒ 1M Tris-HCl pH=7.5 **Invitrogen - Thermo Fisher Catalog #15567-027**

☒ 5M NaCl **Ambion Catalog #AM9760G**

☒ Surfact-Amps NP-40 **Thermo Fisher Scientific Catalog #28324**

☒ UltraPure Distilled Water **Invitrogen - Thermo Fisher Catalog #10977-015**

☒ Albumin, Bovine Serum, 10% Aqueous Solution, Nuclease-Free **Merck MilliporeSigma (Sigma-Aldrich) Catalog #126615-25ML**

☒ Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution in Water **Thermo Fisher Scientific Catalog #H3570**

☒ Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #03335399001**

☒ Kimble™ Kontes™ Dounce Tissue Grinders **Fisher Scientific Catalog #K885300-0015**

STEP MATERIALS

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☒ UltraPure Distilled Water **Invitrogen - Thermo Fisher Catalog #10977-015**

☒ 10x PBS **Thermo Fisher Scientific Catalog #AM9624**

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Protocol materials

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

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

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

⊗ 1M Tris-HCl pH=7.5 **Invitrogen - Thermo Fisher Catalog #15567-027**

⊗ 5M NaCl **Ambion Catalog #AM9760G**

⊗ 1M MgCl₂ **Ambion Catalog #AM9530G**

⊗ Surfact-Amps NP-40 **Thermo Fisher Scientific Catalog #28324**

⊗ Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #03335399001**

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⊗ UltraPure Distilled Water **Invitrogen - Thermo Fisher Catalog #10977-015**

⊗ 10x PBS **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

new equipment

NAME

Fisher Scientific

BRAND

K885300-0015

SKU

15ml Kimble™ Kontes™ Dounce Tissue Grinders

SPECIFICATIONS

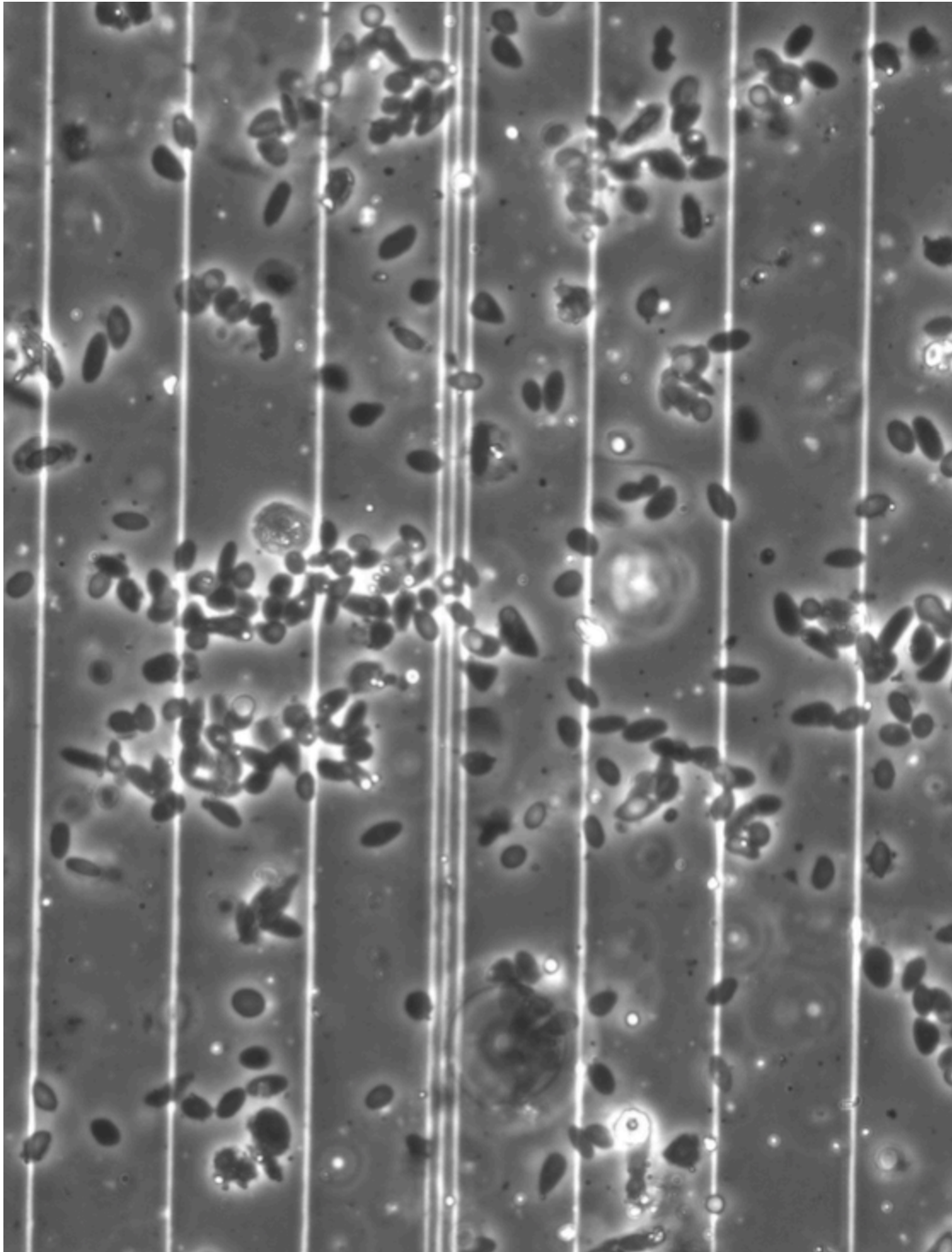
Isolate Nuclei

- 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

new equipment

NAME

Corning

BRAND

352235

SKU

FACS tubes with strainer

SPECIFICATIONS

🧊 4 °C Centrifugation steps should be performed at 4C



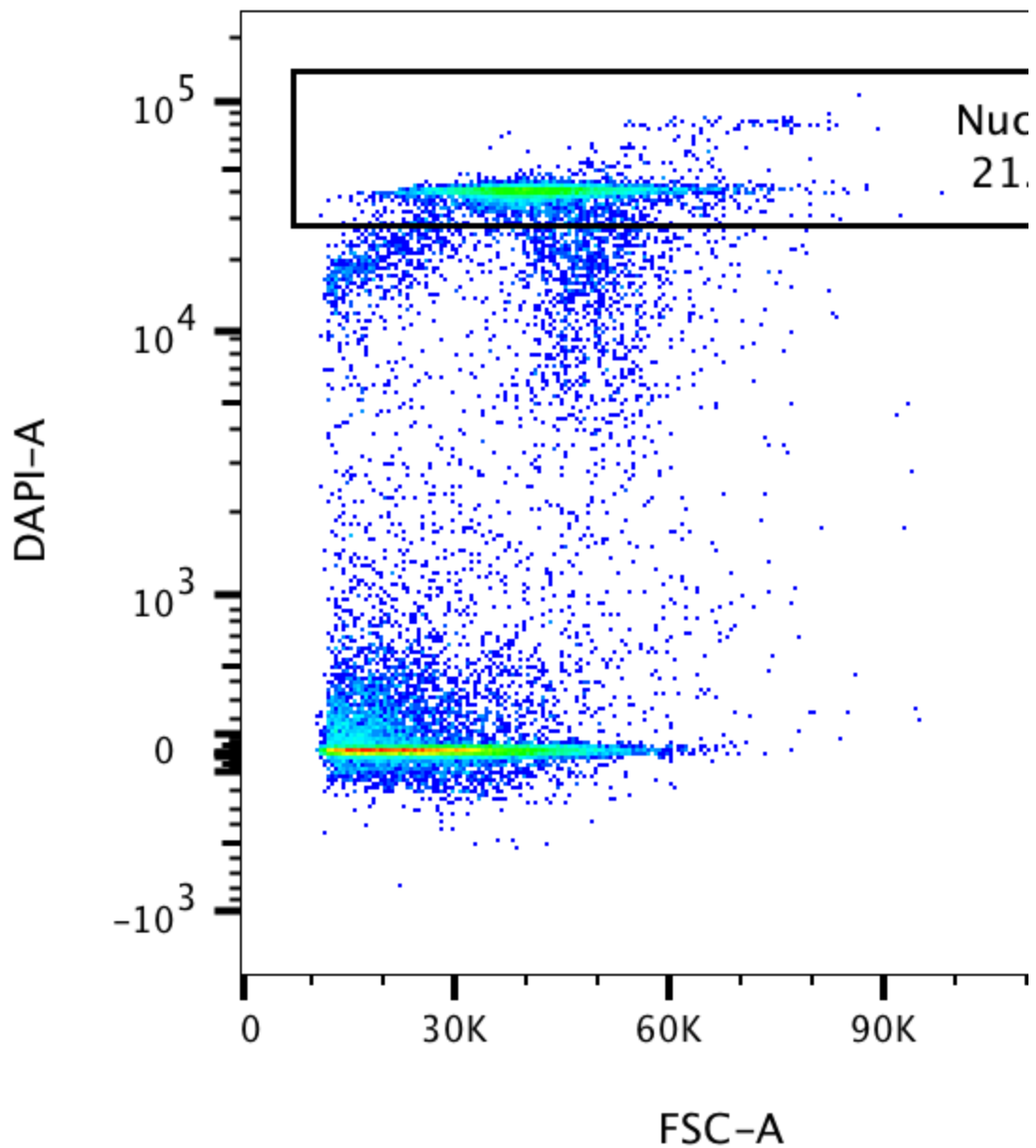
Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution in Water **Invitrogen - Thermo Fisher Catalog #H3570**

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

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

new equipment

NAME

Thermo Fisher

BRAND

AMQAX1000

SKU

Countess II-Automated Cell Counter

SPECIFICATIONS

Expected result



Expected result

