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**14. ABSTRACT****Background**

The development of tumors of the peripheral nervous system (PNS) represents a major problem for persons with Neurofibromatosis type 1. Plexiform neurofibromas (pNFs) constitute a major source of morbidity. pNFs arise during development through the inactivation of the NF1 gene in a cell of the neural crest (NC) - Schwann cell (SC) lineage. In some cases, pNFs may undergo malignant transformation towards an aggressive and highly metastatic Malignant Peripheral Nerve Sheath Tumor (MPNST), normally through the previous development of a pre-malignant nodule termed atypical neurofibroma (aNf). Genomic analyses of pNF-aNF-MPNST progressions have demonstrated that these tumors share the same somatic NF1 inactivation, linking their cells of origin. We don't know whether within pNFs there are remaining cells with the same biological properties as the originating pNF cell, and if so, which role they might play in pNF growth, tumor progression or response to treatment. Our preliminary single cell RNA-seq data from different pNFs confirmed the diversity of cell types present in pNFs, and revealed the existence of cell subpopulations within specific cell type components, a previously unnoticed heterogeneity. The pNF SC component seems to contain at least two distinct groups of SCs, one expressing exclusively markers of precursor SCs (SCPs) and another group expressing in addition markers of SC commitment. There is also heterogeneity in the endoneurial fibroblast-like stromal (FB) component, with some subpopulations expressing key mesenchymal transcription factors also identified in MPNST cells. The FB component might play an important role in pNF growth. We have recently generated human neurofibroma-like tumors in mice by engrafting 3D spheroids in their sciatic nerve. Only when these spheroids contain NF1(-/-) iPSC-derived differentiating SCs plus primary endoneurial FBs neurofibroma-like tumors consistently develop.

**Hypothesis**

We hypothesize that some of the pNF cell populations expressing SCP markers could act as a pNF stem-like cell. We think these cells might have an important impact on pNF growth, progression to malignancy and response to treatment. In addition, we hypothesize that pNF FBs provide trophic and niche conditions essential to maintain this pNF stem-like cell population. Finally, a fine characterization of expression and mutation status at single cell level may uncover the cells undergoing tumor progression. These cells need to be present in faithful models to help characterize the impact of drug treatments on different tumor cell subpopulations.

**Specific Aims**

1) To generate a shared resource consisting in a comprehensive cell diversity map of the cellular composition, spatial distribution and genomic content of NF1-associated peripheral nerve sheath tumors (pNFs, aNFs and MPNSTs). 2) To biologically characterize and elucidate the contribution of specific subpopulation of cells of the SC and FB components, on pNF growth. 3) To identify and characterize the identity of the cell type within pNFs progressing towards aNF and MPNSTs. Analyze the presence of these cells in a human iPSC-based in vitro/in vivo 3D tumor model, and monitor the impact of drug treatment on their viability.

**Study design**

We will generate a resource on single cell information of pNFs, aNFs and MPNSTs available to the scientific community. This resource will provide a comprehensive map at single cell resolution, combining four layers of information: gene expression (scRNA-seq), genetic and genomic content (target scDNA-seq), spatial distribution (spatial transcriptomics) and functional properties. Single cell information will be used to separate and functionally characterize (potency, trophic and niche capacities) specific subpopulations of cells within pNFs cell components. The identification of somatic mutations (NF1, CDKN2A) will be used to trace cells progressing from pNF to aNF and MPNST. We will characterize our iPSC-based 3D tumor model system for the presence of these cells that will be used to monitor tumor response to Selumetinib in a pilot treatment.

**Impact**

The present work may change the way we understand the cell composition and heterogeneity of pNFs. We will better understand the impact of specific cell subpopulations on pNF growth and progression to MPNST. Translating this cell composition information to faithful tumor models may provide a way to analyze tumor response to treatment based on the impact on specific cell

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## **1. INTRODUCTION**

The development of tumors of the peripheral nervous system (plexiform neurofibromas (pNFs); atypical neurofibromas (aNf); malignant peripheral nerve sheath tumor (MPNST)), represents a major clinical problem for persons with Neurofibromatosis type 1 (NF1). Plexiform neurofibromas (pNFs) constitute a major source of morbidity and can progress towards an aggressive and highly metastatic MPNST. The cell originating these tumors and their cellular composition are key aspects to understand how these tumors grow, progress and respond to therapies. In the present project we propose to perform an integrative analysis of different genomic techniques at a single cell level of pNFs, aNFs and MPNSTs, comparing them to nerves, their tissue of origin. We will generate a shared resource consisting in a comprehensive cell diversity map of the cellular composition, spatial distribution and genomic content of NF1-associated peripheral nerve sheath tumors. We will elucidate the contribution of specific subpopulations of cells within these tumors to their growth and will characterize the identity of the cell type within pNFs progressing towards aNF and MPNSTs. Finally, we will analyze the impact of drug treatments on specific subpopulations of cells using a human iPSC-based *in vitro/in vivo* 3D tumor model developed in our lab.

## **2. KEYWORDS**

Plexiform neurofibroma; atypical neurofibroma; malignant peripheral nerve sheath tumor (MPNST); nerve; Schwann cell; fibroblast; cell-of-origin; cell heterogeneity; single cell analysis; genomics; bioinformatics

### **3. ACCOMPLISHMENTS**

Major goals of the project within months 1-12

**As stated in the SOW**

<b>Specific Aim 1: To generate a shared resource consisting in a comprehensive cell diversity map of the cellular composition, spatial distribution and genomic content of NF1-associated peripheral nerve sheath tumors (pNFs, aNFs and MPNSTs) and adult nerves</b>	<b>Timeline (Months)</b>	<b>Site 1 GSU</b>
<b>Major Task 1:</b> Expression analysis: bulk RNAseq, scRNAseq, spatial transcriptomics		
Subtask 1: HRPO review and approval	1-4	
Subtask 2: Bulk RNAseq from primary tumors and nerves Samples used: Human samples of pNFs, aNFs/ANNUBP*, MPNSTs, adult nerve (collected and preserve in our IRB-approved collection of samples number C.0002242 of the National Biobank Registry of Instituto de Salud Carlos III, Spain). (*) aNFs/ANNUBP are partially being collected prospectively. We cannot guarantee the proposed timeframe for this specific tumor type. At least 4 samples of each. R1, R2	4-8	X
Subtask 3: Bulk RNAseq bioinformatic analysis	8-12	X
Subtask 4: scRNAseq from primary tumors and nerves Samples used: Same samples used for Subtask 1 At least 4 samples of each. R1, HH	4-8	X
Subtask 5: scRNAseq bioinformatic analysis	8-12	X
Subtask 6: Smart-seq from primary tumors and nerves Samples used: Same samples used for Subtask 1 At least 4 samples of each. R1, R2, HH	4-12	X
Subtask 7: Smart-seq bioinformatic analysis	8-18	X

What has been accomplished

<b>Specific Aim 1: To generate a shared resource consisting in a comprehensive cell diversity map of the cellular composition, spatial distribution and genomic content of NF1-associated peripheral nerve sheath tumors (pNFs, aNFs and MPNSTs) and adult nerves</b>	<b>Timeline (Months)</b>	<b>Site 1 GSU</b>
<b>Major Task 1:</b> Expression analysis: bulk RNAseq, scRNAseq, spatial transcriptomics		
Subtask 1: HRPO review and approval	1-4	
<b>Achieved</b>		
Subtask 2: Bulk RNAseq from primary tumors and nerves  Samples used: Human samples of pNFs, aNFs/ANNUBP*, MPNSTs, adult nerve (collected and preserve in our IRB-approved collection of samples number C.0002242 of the National Biobank Registry of Instituto de Salud Carlos III, Spain).  (*) aNFs/ANNUBP are partially being collected prospectively. We cannot guarantee the proposed timeframe for this specific tumor type.  At least 4 samples of each. R1, R2	4-8	X
<b>Partially achieved</b>  We have performed bulk RNAseq from 4 pNFs and 4 MPNSTs  We have collected 3 aNF. As stated in the original SOW, these tumors are difficult to obtain. Accordingly, we have prioritized to perform scRNA-seq and SMART-seq analysis, before bulk RNA-seq, since this information was the most crucial to obtain for the project. Now that we confirmed that both single cell techniques work correctly in this type of samples, we will use the remaining parts of the tumors to perform bulk RNAseq.  We have been collecting nerve samples and setting up conditions to dissociate them in order to perform at the same time both bulk RNAseq and single cell RNAseq (see subtask 4 for protocols details).		
Subtask 3: Bulk RNAseq bioinformatic analysis	8-12	X
<b>Partially achieved.</b>  We have designed and implemented the main RNAseq data analysis pipeline.  We have processed the first 8 available samples.  We are designing and implementing the analysis strategy for the integration of RNA-seq and single cell-based technologies		

<p>Subtask 4: scRNAseq from primary tumors and nerves</p> <p>Samples used: Same samples used for Subtask 1</p> <p>At least 4 samples of each. R1, HH</p>	4-8	X
<p><b>Partially achieved</b></p> <p>Preparing the samples for single cell gene expression analysis is critical for maintaining cell integrity and preserving each cell's transcriptome. It is critical to obtain a well-cingulated cell suspension free of cell debris, with minimal cell aggregates and high viability (&gt;70%). In general, cell preparation protocols vary depending on the tissue of origin. Each tissue type is unique and thus, it is critical to optimize sample preparation before starting single cell experiments.</p> <p>We have set up conditions and established robust protocols to process all tumor types (pNFs, aNFs and MPNSTs) and also nerves, for single cell RNA-seq analysis, for either 10x scRNA-seq and SMART-seq. These protocols include a dissociation step specific for each type of tissue that maintains cell integrity, a removal of cell debris and aggregates step by using the Death Cell Removal kit (Miltenyi Biotec); a removal of myelin step for nerve samples (Miltenyi Biotec), and a final confirmation step of high cell viability using Flow cytometry. We below the section "<u>Developed Protocols</u>".</p> <p>We prioritized to set up the whole single cell analysis workflow (sample dissociation; single cell preparation; library constructions; sequencing; bioinformatic analysis) of few samples of different tumor/tissue types first, instead of performing sample preparation and sequencing for all samples before checking that the whole workflow was performing as expected.</p> <p>We performed scRNAseq from 2 pNFs, 3 aNFs and 3 MPNSTs, and are currently performing the remaining samples, that will be finished at the end of the summer.</p>		
<p>Subtask 5: scRNAseq bioinformatic analysis</p>	8-12	X
<p><b>Partially achieved</b></p> <p>As stated above, we prioritized having produced and analyzed a small number of pilot samples for the complete workflow, rather than performing only the sequencing part for all the samples upfront. Therefore, although we implemented the whole scRNAseq data analysis pipeline, we put a special emphasis on the quality control (QC) and batch effect analysis code.</p> <p>In particular:</p> <p>We have designed and implemented the infrastructure for <b>scRNAseq data analysis</b> including a data analysis pipeline with:</p> <ul style="list-style-type: none"> <li>- Standard well tested preprocessing and QC steps taking into account the most frequent error and noise sources found from initial library</li> </ul>		

preparation to the final sequencing.

- Additional extended QC for the detection of cellular stress prior to library preparation. We have identified cellular stress as a major noise source in a few samples and developed a new bioinformatics methodology to identify stressed cells and samples and correct the problem. Additional information can be found below in the section entitled "Extended QC for stress detection and removal".

- The analysis of batch effects and the application of corrective batch effect removal measures to ensure full comparability between all samples in the project. Our results so far show differences between individual samples but not strong batch effects.

- Single-cell level cell-type identification with a two-tier cell-type assignment and analysis of sample-level cell-type proportions. We are still testing different reference single cell atlases to identify the one performing the best.

- Extensive gene expression analysis, from cluster-specific marker gene identification to individual gene expression evaluation and differential expression analysis between any groups of cells from the same or different samples.

- Gene-set expression analysis for the integrated analysis of the expression patterns of complete sets of genes.

- A specialized method based on multiple gene-set expression analysis to identify cells at different differentiation stages along the Neural Crest-Schwann Cell axis, from neural-crest to mature Schwann cells.

As it will be explained in the section "Changes/Problems", in order to make this single cell data set even more complete, we decided to include single cell epigenetic information generated in the context of another project, but for the same samples analyzed here.

We have designed and implemented the infrastructure for scATAC-seq data analysis including:

- Preprocessing, mapping, basic peak calling and integration of multiple samples.

- Additional peak refinement and peak-gene assignment.

- Differential accessibility analysis between any group of cell from the same or different samples.

- Analysis of enhancer activation/deactivation based on the accessibility of known enhancers.

- ATAC-based clustering and identification of cluster-level accessibility markers

<p>- Integration of scATAs-eq with scRNA data including gene-level accessibility analysis based on cell clusters defined from scRNAseq data</p> <p>In addition to this, we have set up a specific pipeline for RNA-velocity based on spliced/unspliced transcripts detected in scRNAseq data and the integration of these results with the main scRNAseq and scATACseq pipelines.</p> <p>Finally, we are working on the estimation of cell-level broad copy-number profiles based on scRNAseq data, especially useful for atypical neurofibromas and MPNST.</p> <p>A short summary of a few analysis results and very-preliminary findings can be found below in the section entitled "<u>Observations derived from the preliminary analysis of 4 pNFs and 3 aNFs</u>".</p>		
<p>Subtask 6: Smart-seq from primary tumors and nerves</p> <p>Samples used: Same samples used for Subtask 1</p> <p>At least 4 samples of each. R1, R2, HH</p>	4-12	X
<p><b>Partially achieved</b></p> <p>The samples processed for scRNAseq have been prepared at the same time for SMARTseq analysis. We have established a protocol to FACS-sort the dissociated cells from Subtask 4 in 96-well plates containing the lysis buffer from the SMARTseq preparation kit, provided by CNAG. So far, all quality controls are OK. The quantity of cDNA obtained is adequate and at a good concentration and size. Library preparation has been performed.</p> <p>We have performed SMARTseq from 2 pNFs, 3 aNFs and 3 MPNSTs</p>		
<p>Subtask 7: Smart-seq bioinformatic analysis</p>	8-18	X
<p><b>Partially achieved</b></p> <p>We have designed and set up a very basic data analysis pipeline, but so far we have not been able to test it with our own data and therefore identify specific challenges or steps that need to be fine-tuned.</p>		

## Developed Protocols

<b>Serra Lab Protocols</b>	<b>Version 1</b>	<b>Version 2</b>
<b>IGTP</b>	<b>Prepared by: H. Mazuelas</b>	<b>Revised by: M.Carrió</b>
	<b>Date: January 2020</b>	<b>Date: July 2022</b>

SOP nº8

### **PNF, ANNUBS (ANFs) and MPNST dissociation for scRNAseq**

#### **Objective**

To dissociate PNFs, ANNUBS (ANFs) and MPNSTs for single cell RNAseq analysis

#### **Materials and Reagents**

Collagenase Type I (Worthington Cat# LS004196)  
Neutral Protease (Dispase grade I) (Worthington Cat# LS02104)  
Normocin cocktail (Invivogen Cat# ANT-NR-1)  
DMEM (Gibco 2196035)  
FBS (Gibco 10270106)  
GlutaMax (Glx) (Gibco 35050038)  
Penicillin/Streptomycin (P/S) (Gibco 15140122)  
Dead Cell Removal Kit (Miltenyi Biotec 130-090-101)  
MACS MS columns (Miltenyi Biotec 130-042-201)  
MACS Multistand ( Miltenyi Biotec 130-042-303)  
MACS separator (Miltenyi Biotec)

#### **Methodology**

Note: Samples need to be delivered at CNAG before 13:00h

To prepare:

- Dissociation media :
  - 160 U/ml Collagenase Type I
  - \*dilution 1:2 Collagenase lot 49HI9435 for PNF and ANNUB
  - \*dilution 1:5 Collagenase lot 49HI9435 for MPNST
  - 0.8 U/ml Neutral Protease (Dispase grade I)
  - 1x Normocin cocktail
  - DMEM 10%FBS +1xGlx +1xP/S
  - Mix and filter using a sterile 0,22µm PES filter unit

#### **10 mL 1:2 Collagenase (PNF/ANNUB)**

0,5 mL Collagenase type I (1600 U/mL stock)  
260µl Dispase (30 U/mL stock)  
20µl normocin  
9,2 mL DMEM 10%FBS +1xGlx +1xP/S  
Mix and filter using a sterile 0,22µm PES filter unit

### **10 mL 1:5 Collagenase (MPNST)**

0,2 mL Collagenase (1600 U/mL stock)

260µl De dispase (30 U/mL stock)

20µl normocin

9,5 mL DMEM 10%FBS +1xGlx +1xP/S

Mix and filter using a sterile 0,22µm PES filter unit

- Binding buffer 1X from Dead Cell Removal Kit (in cell culture H<sub>2</sub>O; prepare 3,5mL/sample + 0,5mL more to calibrate the column)
  - o 10 mL binding buffer: 500 µl 20x binding buffer + 9,5 mL H<sub>2</sub>O
- Prepare 15 ml conical centrifuge tubs to collect samples, SN1, SN2 for each tumor to dissociate
- Prepare 15 ml conical centrifuge tubs to collect alive cells (negative fraction)
- Prepare 15 ml conical centrifuge tubs to collect death cells (positive fraction. OPTIONAL)
- Prepare 1,5ml Low Binding Eppendorf's to aliquot cells to take to CNAG

#### Protocol:

##### Dissociation

- Transfer the piece of tumor into a sterile culture dish containing some drops of dissociation media and cut it into 2 mm x 2 mm pieces.
- Place 4-8 neurofibroma pieces per well of a 6-well plate and add 2 mL of dissociation buffer and incubate in a standard CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub> for 16h
- Next day, mechanically dissociate the neurofibroma tissue into a suspension using a glass Pasteur pipette. Observe the sample under the microscope:
  - o If there are aggregates, place the sample at 37 °C for an extra hour.
  - o If the sample is in single cell suspension proceed with the protocol
- Transfer the neurofibroma suspension into a 15 mL conical centrifuge tube and add 8 mL complete DMEM to stop the activity of dissociation enzymes.
- Centrifuge at 1400 rpm 5 min.
- Recover supernatant (SN1) and repeat the centrifugation step. Keep the pellet of cells apart.
  - o The reason of this step is because sometimes many cells remain in the supernatant.
- Centrifuge SN1 at 1400 rpm 5 min.
  - o If the supernatant 2 (SN2) is still cloudy, you can centrifuge SN2.
- Resuspend pellet 1 and pellet 2 in 0,5-1 mL (depending on the pellet) in DMEM 10% FBS +1xGLx + 1x P/S
- Count cells

**IMPORTANT:** For PNFs that they contain a lot of fiber, if the number of cells counted is more than 3M, use two columns in the following steps to prevent collapsing them.

**IMPROTANT:** If the cell pellet is very small, DO NOT count the cells and go straight to perform Death Cell Removal.

- Centrifuge at 1200 rpm 5 min
- Discard supernatant using a pipette tip.

##### Dead Cell Removal Kit (Miltenyi Biotec 130-090-101)

- Add 100 µl Dead Cell Removal Microbeads (enough for 10M cells).
- Incubate 15 min at room temperature (protected from light).

### Preparation of MS MACS columns MACS

- Mount columns in the MACS separator attached to the MACS Multistand (avoid position 1 )
- Equilibrate columns with 500 µl Binding buffer 1x

### Magnetic separation

- Add 500µl Binding buffer 1x to the sample containing the Microbeads
- Apply cell suspension into the column

**IMPORTANT:** if the column collapses, take the column and transfer it to a new equilibrated column.

- Collect alive cells (negative fraction) into a 15 mL conical centrifuge tube
- Wash the column with 4 x 500 µl Binding Buffer 1x
- Count alive cells: take 10 µl from the total volume of 2,5 mL without making any dilution
- Centrifuge alive cells at 1200 rpm 5 min
- Resuspend cells at concentration of 1.000 cells/ µL (1.000.000 cells/ mL) in **DMEM+10%FBS+Glx w/o antibiotic**.
  - o Optional: Take column from the Multistand, add 1mL of 1x binding buffer and elute positive fraction of cells (death cells) into a 15 mL tube. Observe cells under a Neubauer chamber.

### Sample preparation to take to CNAG

- Divide the sample for scRNAseq\_ATAqseq (Multiome) (CNAG) and SMARTseq (FACS-sorting) into two low binding Eppendorf's:
  - o Multiome: 0,5-1M cells
  - o SMARTseq: Minimum 50.000 cells
- Place sample on ice and go:
  - o Multiome: to CNAG straight away
  - o SMARTseq: start de staining with DAPI to sort the cells (See SOP9).

<b>Serra Lab Protocols</b>	<b>Version 1</b>	
<b>IGTP</b>	<b>Prepared by: M.Carrió</b> <b>Date: March 2022</b>	<b>Revised by: M.Carrió</b> <b>Date: July 2022</b>

SOP nº9

## Sample preparation for SMARTseq

### Objective

To prepare single-cell samples (from SOP8, SOP10) and FACS-sort them in 96 well plates for SMARTseq.

### Materials and Reagents

SMARTseq2 plates (provided by CNAG)

DMEM 10%FBS 1xGlx No P/S

PBS

DAPI

### Methodology

Tissues are dissociated for scRNAseq analysis following specific protocols (SOP8, SOP10)

Cells are resuspended at a concentration de 1.000 cells/ $\mu$ L (1M cells/ml) in DMEM+10%FBS+Glx -P/S

- Divide the sample in two:
  - o For Multiome: 0,5-1M cells
  - o For SMARTseq: Minimum 50.000cells

#### SMARTseq cell preparation:

Take an aliquot of cells and stain them with DAPI to asses cell viability

Ideal situation:

- 100.000 cells or more:
  - 10.000 DAPI staining to assess viability
  - The rest for sorting

- 50.000 cells:
  - 10.000 DAPI
  - The rest for sorting

Keep the rest of the cells to be sorted on ice

DAPI staining:

- Prepare DAPI solution (1:1000 in PBS)
- Centrifuge cells 300g 3min RT
- Add 100  $\mu$ L DAPI solution
- Incubate 10 min at RT
- Add 900  $\mu$ L PBS
- Centrifuge cells 300g 3 min RT
- Resuspend cells in 200  $\mu$ L DMEM+10%FBS+Glx-P/S

Go to cytometry facility

Prepare plate for sorting

- Thaw SMART-seq2 plates on ice (follow CNAG instructions for preparing the plate).
- NAME THE PLATE:
  - o Ex: SERRA\_14 P2 DATA
    - SERRA\_14 (project name) P2 (plate 2) Date
- Centrifuge the plate 2000 rpm 1min 4°C (use centrifuge located at genomic facility)

- Keep plate on ice until sorting

Sort cells:

- Analyze cell viability using DAPI-stained cells
- Remove plate cover from the plate
- Sort non-stained cells into SMART-seq2 plates (follow CNAG suggestions for plate layout).
- Place a new plate cover (provided by CNAG)
- Once the sorting is finished, centrifuge the plate 2000 rpm 1 min 4°C
- Snap freeze the plate on dry ice
- Store at -80 °C until shipment to CNAG

<b>Serra Lab Protocols</b>	<b>Version 1</b>	
<b>IGTP</b>	<b>Prepared by: MCarrio</b>	
	<b>Date: May 2022</b>	

SOP nº10

## **Nerve dissociation for scRNAseq**

### **Objective**

To dissociate nerves for single cell RNAseq analysis.

### **Materials and Reagents**

Collagenase Type I (Worthington Cat# LS004196)  
 Neutral Protease (Dispase grade I) (Worthington Cat# LS02104)  
 Normocin cocktail (Invivogen Cat# ANT-NR-1)  
 DMEM (Gibco 2196035)  
 FBS (Gibco 10270106)  
 GlutaMax (Glx) (Gibco 35050038)  
 Penicillin/Streptomycin (P/S) (Gibco 15140122)  
 Dead Cell Removal Kit (Miltenyi Biotec 130-090-101)  
 MACS MS columns (Miltenyi Biotec 130-042-201)  
 MACS Multistand ( Miltenyi Biotec 130-042-303)  
 MACS separator (Miltenyi Biotec)  
 Myelin Removal kit (Miltenyi Biotec 130-096-731)  
 70 µm Flowmi Cell strainer (Sigma BAH136800070)

### **Methodology**

**NOTE:** Samples need to be delivered at CNAG before 13:00h

To prepare:

- Dissociation media:
  - 160 U/ml Collagenase Type I
  - 0.8 U/ml Neutral Protease (Dispase grade I)
  - 1x Normocin cocktail
  - DMEM 10%FBS +1xGlx +1xP/S
- Mix and filter using a sterile 0,22µm PES filter unit

#### **10 mL**

- 1 mL Collagenase (1600 U/mL stock)
  - 260 µL Dispase (30 U/mL stock)
  - 20µl normocin
  - 9,2 mL DMEM 10%FBS +1xGlx +1xP/S
- Wash buffer for Myelin Removal beads: PBS + 0,5% FBS (COLD)

- Binding buffer 1X from Dead Cell Removal Kit (in cell culture H<sub>2</sub>O; prepare 3,5mL/sample + 0,5mL to calibrate the column)
  - o 10 mL binding buffer: 500 µl 20x binding buffer + 9,5 mL H<sub>2</sub>O
- Prepare 15 mL conical centrifuge tubs to collect life cells (negative fraction)
- 1,5ml Low Binding Eppendorf's to aliquot cells to take to CNAG

#### Protocol:

##### Dissociation

- Transfer a piece of peripheral nerve into a sterile tissue culture plate with one drop of dissociation media.
- Cut the nerve longitudinally and try to pull the fascicles out of the epineurium.
- Cut the fascicles into 2 mm x 2 mm fragments and transfer them into a 6-well plate with 2 mL of dissociation media per well.
- Incubate at 37°C 16 h.
- Next day, mechanically dissociate the nerve tissue into suspension using a glass Pasteur pipette. Observe the sample under the microscope:
  - o If there are aggregates, place sample at 37 °C for an extra hour
- Repeat this procedure every 30 min until single cell suspension is obtained (around 17-18 h a 37 °C with Pasteur pipette help).
- Transfer the sample suspension into a 15 mL conical centrifuge tube and add 8 mL complete DMEM to stop the activity of dissociation enzymes.
- Centrifuge at 1400 rpm 5 min.
- Recover supernatant (SN1) and repeat the centrifugation step. Keep the pellet of cells apart.
  - o The reason of this step is because sometimes many cells remain in the supernatant.
- Centrifuge SN1 at 1400 rpm 5 min
  - o If supernatant 2 (SN2) is still cloudy, you can centrifuge SN2
- Resuspend pellet 1 and pellet 2 in 0,5-1 mL (depending on the pellet) in complete DMEM.
- Filter sample using a 70 µm Flowmi Cell strainer
- Count cells using a hemocytometer
- **IMPORTANT:** If the cell pellet is very small, we DO NOT count the cells and go straight to perform Myelin Removal.
- Centrifuge 1400 rpm 5 min

##### Myelin Removal

- Add 180µl buffer (PBS + 0,5%FBS) COLD + 20µl myelin removal beads to the cell pellet.
- Incubate 15 min 4 °C (protected from light).

##### Preparation of MS MACS columns MACS

- Mount columns in the MACS separator attached to the MACS Multistand (avoid position 1 )
- Equilibrate columns with 500 µl Binding buffer 1x

##### Magnetic separation

- Add 2 mL cold buffer to the cells and beads.
- Centrifuge 1400 rpm 5 min.
- Aspirate supernatant.
- Resuspend in 1 mL of buffer.
- Apply cell suspension into the column.
- Wash the column with 4 x 500 µl Buffer

### Dead Cell Removal (Milteny Biotec 130-090-101)

- Centrifuge at 1200 rpm 5 min
- Discard supernatant using a pipette tip.
- Add 100 µl Dead Cell Removal Microbeads (enough for 10M cells).
- Incubate 15 min at room temperature (light off).

### Preparation of MS MACS columns MACS

- Mount columns in the MACS separator attached to the MACS Multistand (avoid position 1 )
- Equilibrate columns with 500 µl Binding buffer 1x

### Magnetic separation

- Add 500µl Binding buffer 1x to the sample containing the microbeads
- Apply cell suspension into the column

**IMPORTANT:** if the column collapses, take the column and transfer it to a new equilibrated column.

- Collect alive cells (negative fraction) into a 15 mL conical centrifuge tube
- Wash the column with 4 x 500 µl Binding Buffer 1x
- Count alive cells: take 10 µl from the total volume of 2,5 mL without making any dilution
- Centrifuge alive cells at 1200 rpm 5 min
- Resuspend cells at concentration of 1.000 cells/µL(1.000.000 cells/mL) in **DMEM+10%FBS+Glx w/o antibiotic**.
  - o Optional: Take column from the magnetic rack, add 1mL of 1x binding buffer and elute positive fraction of cells (death cells) into a 15 mL tube. Observe cells under a Neubauer chamber.

### Sample preparation to take to CNAG

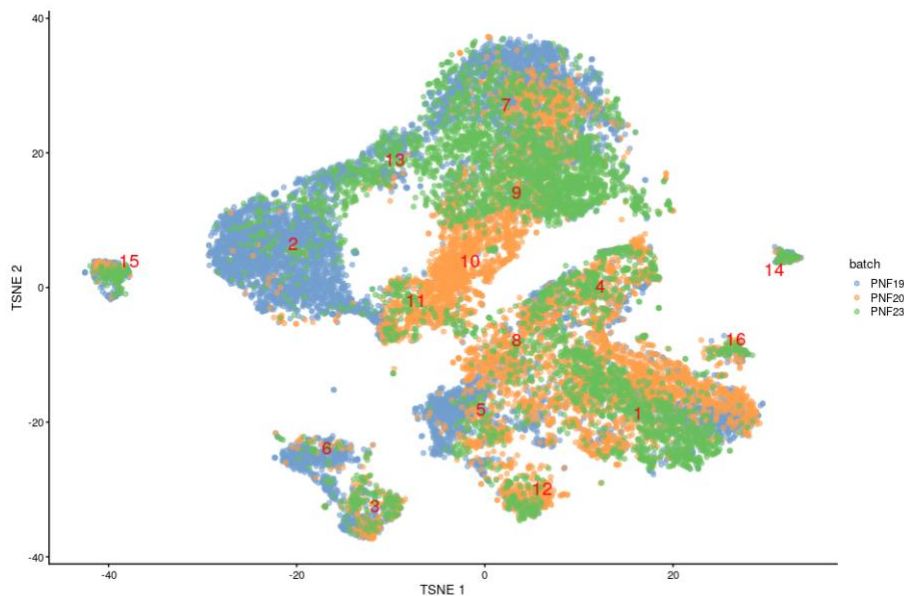
- Divide the sample for scRNAseq\_ATAqseq (Multiome) (CNAG) and SMARTseq (sorting) into two low binding Eppendorf's:
  - o Multiome: 0,5-1M cells
  - o SMARTseq: Minimum 50.000 cells
- Place sample on ice and go:
  - o Multiome: to CNAG straight away
  - o SMARTseq: start de staining with DAPI to sort the cells (See SOP9).

## Extended QC for stress detection and removal

Quality control (QC) is an integral part of any data analysis pipeline. In the case of scRNAseq data, there exist extensive QC options taking into account most frequent sources of noise and technical variability in the process from library preparation to sequencing, that is, from the moment the sample enters the Chromium Controller to the generation of sequencing data. This QC and normalization steps include adjusting for the total sequencing depth of each sample, detecting and removing empty GeMs, detecting and removing doublets, detecting and removing GeMs with dying cells or just cell death debris and many other factors affecting the quality of the scRNAseq data.

However, in our datasets we identified a different source of sample variability that could greatly affect the analysis and conclusions: cellular stress. We detected that some of the samples presented a subset of cells with very high stress levels, in our case Endoplasmatic Reticulum stress. This stress had such an impact on the the global transcriptional profile of these cells that dominated their positioning in the dimensionality reduction plots.

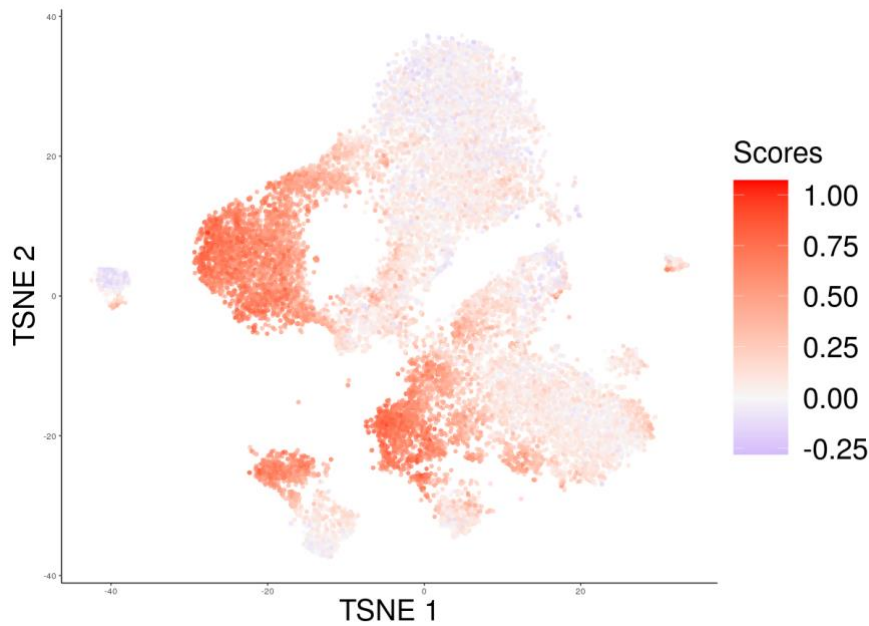
For example when analyzing the first three PNF samples, we can see some regions with only cells from PNF19 (Figure 1). This has the effect of creating additional clusters that could be interpreted as different cell types or the tumor having cells in a special state.



**Figure 1 – Stress affects the positioning of cells in the dimensionality reduction plots.** We can see how some parts of the TSNE plot show cells from the PNF19 (blue) and create new additional cell clusters (red numbers).

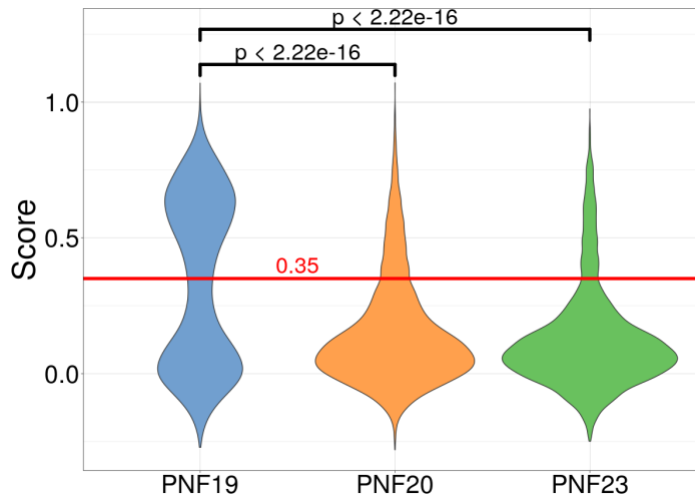
We developed a system leveraging stress-related gene-sets from gene ontology with the possibility of computing a score for each cell describing the expression level of a set of genes. With this, we can identify the stress levels of each cell and plot them in the dimensionality reduction plots.

In the example above, we can see how each of the regions containing only PNF19 cells is highly enriched in stressed cells (Figure 2).



**Figure 2 – Stress levels at single cell resolution.** The expression score of the set of genes associated to endoplasmatic reticulum stress shows a concentration of high scoring (stressed, in red) cells in the same regions that contained only PNF19 cells.

We studied the distribution of stress scoring for each sample and saw how PNF19 had a significant accumulation of cells with high stress scores. (Figure 3)

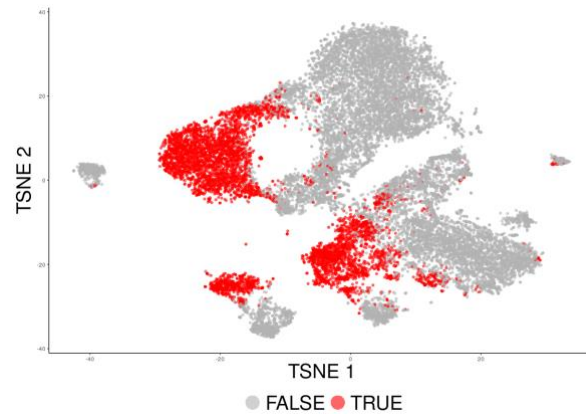


**Figure 3 – Stress levels per sample.** The distribution of the scoring values for each sample show an accumulation of high scoring cells in PNF19 with respect to the other two samples.

We think the stress levels are most likely not due to some intrinsic property of the tumor but to variations in the sample processing once extracted by the surgeons. Maybe the time elapsed between the tumor resection and the deposition of the specimen in the tube with media was different, or maybe there were differences in temperatures along the process, or any other environmental factor affecting the tumor.

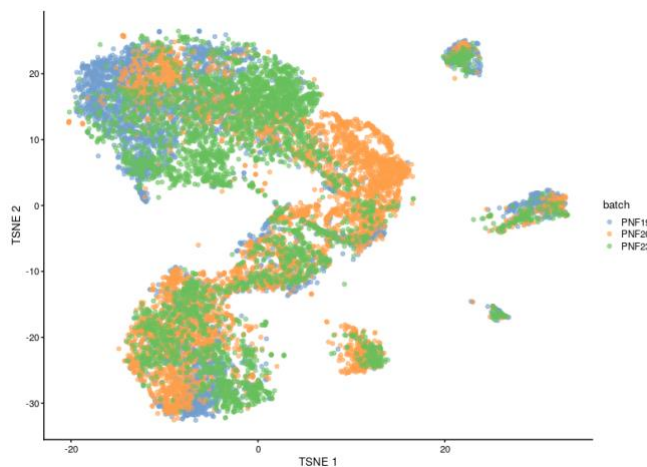
In any case, we decided that the best course of action was to completely remove the stressed cells instead of trying to correct the stress signature by statistical means, given how the stress affected in unknown ways a large number of genes.

Therefore, we set a scoring threshold (Figure 3) and removed all cells from all samples above that threshold (Figure 4).



**Figure 4 – Removed cells.** Cells with the stress scoring level above the threshold (red) were removed from the analysis.

After removing the stressed cells we performed again the clustering and dimensionality reduction steps and obtained a much more informative image, which was used for all subsequent analysis. In this new image we can clearly see the two main components (Schwann cells and fibroblastic/mesenchymal) (Figure 5).



**Figure 5 – Reprocessed non-stressed cells.** After the removal of the stressed cells, the TSNE plot changed producing a much more expected distribution.

All in all, we think the identification of stressed cells is an important step in the processing and QC of scRNAseq data that has been largely missing in standard analysis so far. Our pipeline contains a step testing for this and other kinds of cellular stress and has the possibility of removing the stressed cells. We are working on a new software package to facilitate this kind of analysis and our intention is to publish an additional bioinformatics-oriented journal article describing this finding and the software package.

## Observations derived from the preliminary analysis of 4 pNFs and 3 aNFs

We performed a preliminary analysis of the 4 samples sequenced (3 ANFs and 1 PNF) together with the 3 PNF samples previously generated as preliminary data using the scRNAseq data analysis pipeline.

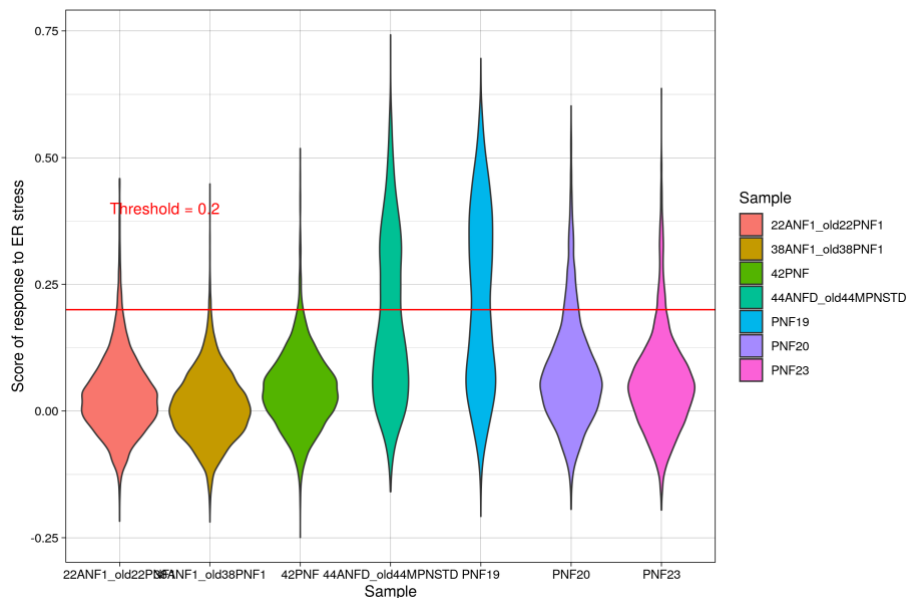
A few observations we deemed scientifically interesting follow:

**Note:** It is important to bear in mind that these are just mere observations and that a rigorous analysis and exploration is needed before any conclusion can be drawn.

### **A – There are more samples with a significant amount of stress.**

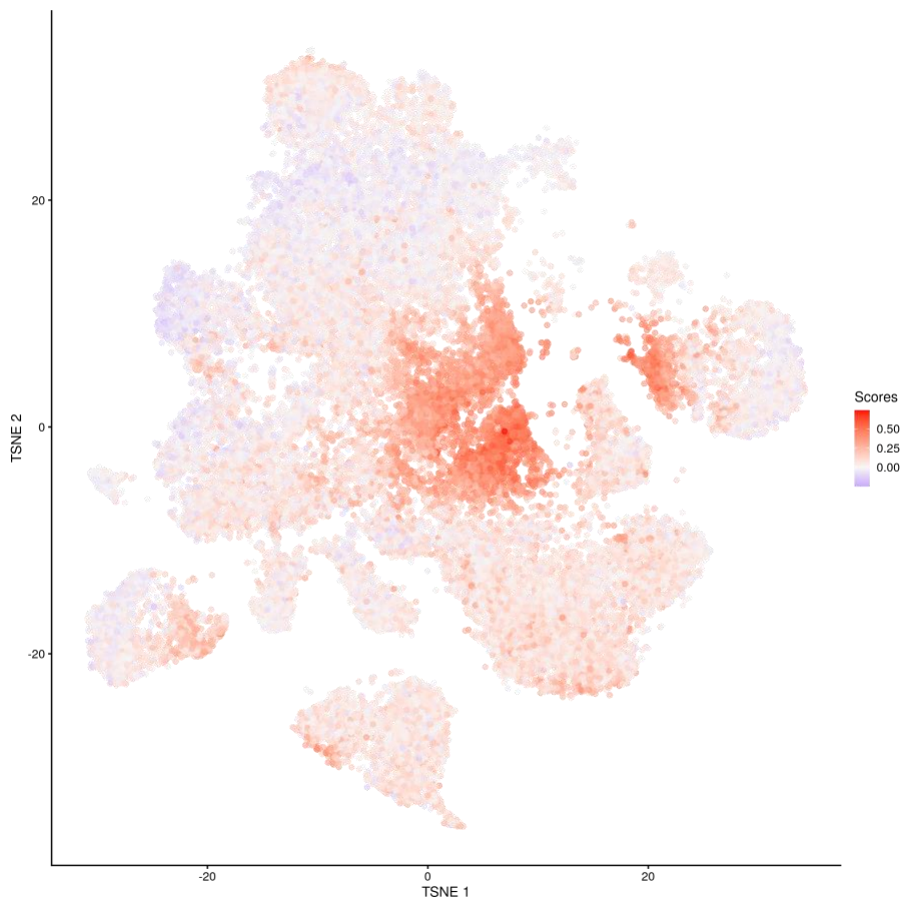
When analyzing the cellular stress levels of all 7 samples we identified 2 samples with a significant amount of stressed cells, PNF19 (seen before in previous section) and 44ANFD. Both samples have a very similar stress profile, while the other 5 samples share a similar low stress profile (Figure 6).

**NOTE:** the actual values and the threshold levels are different than described in the previous section because this data is the scRNAseq part of the Multiome analysis.



**Figure 6 – Stress affects the positioning of cells in the dimensionality reduction plots. We can see how some parts of the TSNE plot show cells from the PNF19 (blue) and create new additional cell clusters (red numbers).**

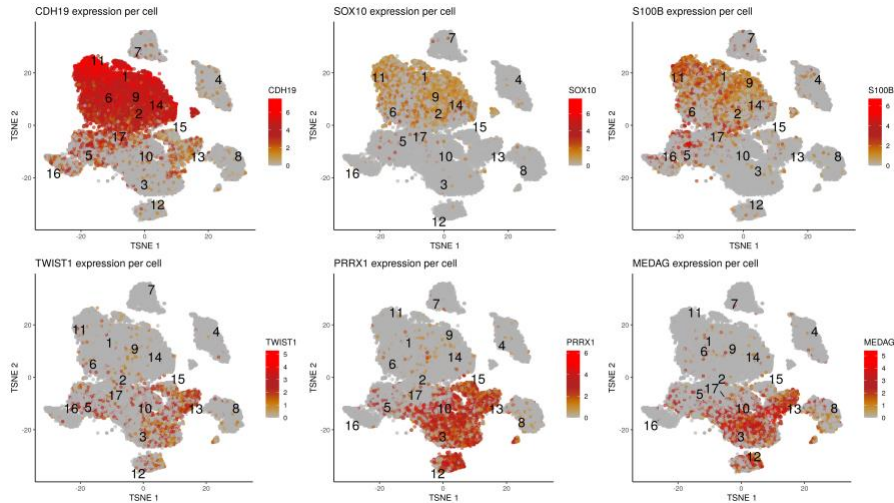
As in the analysis described in the previous section, the stressed cells clustered together and were placed together in the dimensionality reduction plots, showing the impact of the stress in the global transcriptomic profile (Figure 7).



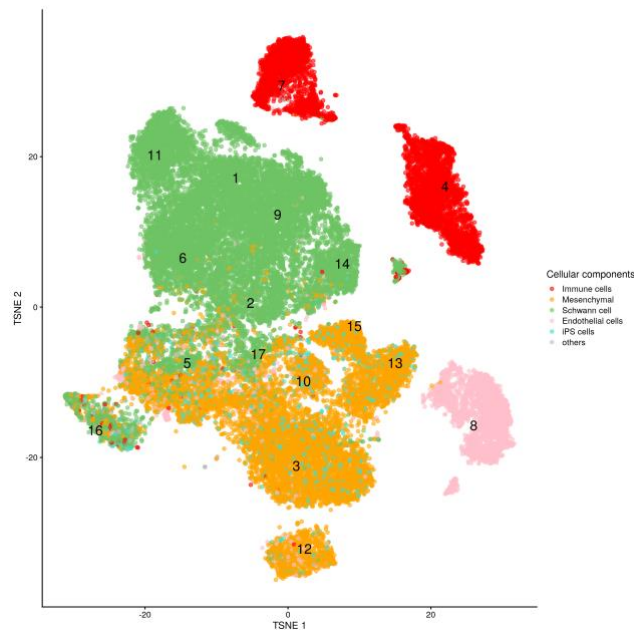
**Figure 7 – Global impact of stress in the transcriptomic profile.** Cells with high stress score cluster together in this TSNE plot, almost regardless of their actual cell-type. This shows the impact of the high stress levels in the transcriptional profile.

## B – The observed cell components are the expected ones, but there are important differences between samples

The main cell components identified by marker gene expression (Figure 8) and single-cell level cell type assignment (Figure 9) showed the expected main cellular components and cell types: Schwann-cell component (the tumor cells), mesenchymal component (the main part of the microenvironment), as well as the endothelial cells and the immune infiltrate.



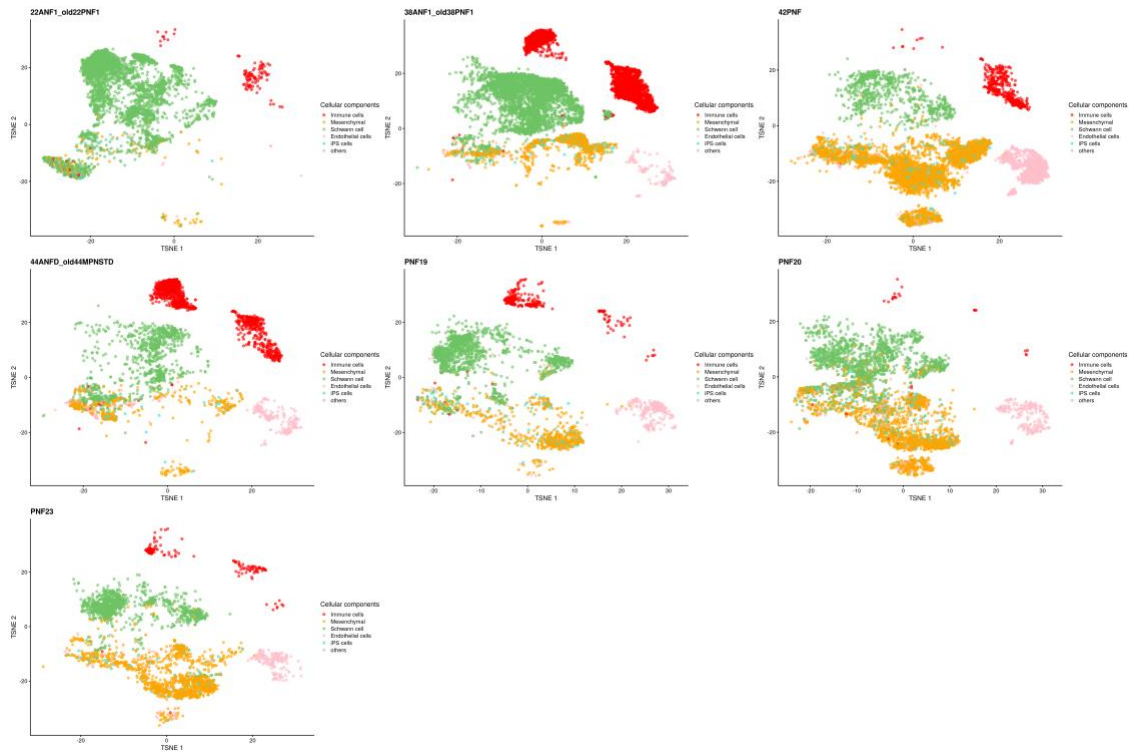
**Figure 8 – Main cell component by marker genes.** The expression of marker genes for the Schwann cell component (top row) and mesenchymal component (bottom row) clearly identifies them. Markers for the other minor components no shown.



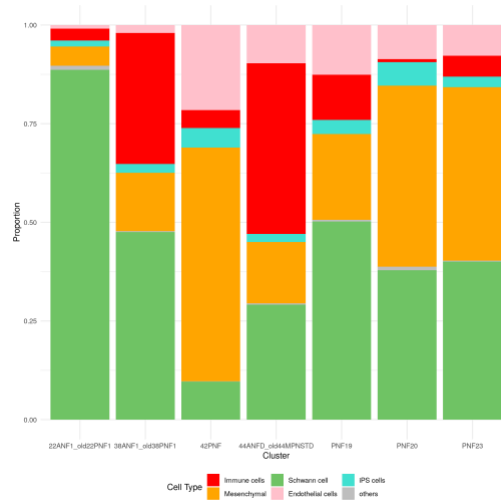
**Figure 9 – Main cell component by cell type assignment.** The components are also clearly recovered by cell-type assignment based on a reference dataset (Human Primary Cell Atlas).

However, although the whole dataset perfectly recovers the expected components in the expected proportions we can see quite striking differences in cellular composition between the different samples (Figure 10 and Figure 11). We can clearly see how ANFs (22ANF, 38ANF and 44ANF) have a lower proportion of mesenchymal cells when compared to PNFs (42PNF, PNF19, PNF20 and PNF23), with the most extreme

example in 22ANF, which is almost completely depleted from mesenchymal cells and endothelial cells and has a very small amount of immune cells. The other two ANFs show a reduced presence of mesenchymal cells but much higher immune infiltrate, which is missing (totally or partially) in the different PNFs. It is important to note that some of these differences may be related to the exact part of the tumor sampled, but the difference in the presence of mesenchymal cells between ANFs and PNFs seems consistent so far and is a biologically plausible result of the expansion of Schwann cells with no CDKN2A in ANFs.



**Figure 10 – Main cell components per sample.** Different samples present different cell-type composition. These plots show the TSNE representation of each sample with each cell colored according to its assigned cell-type.

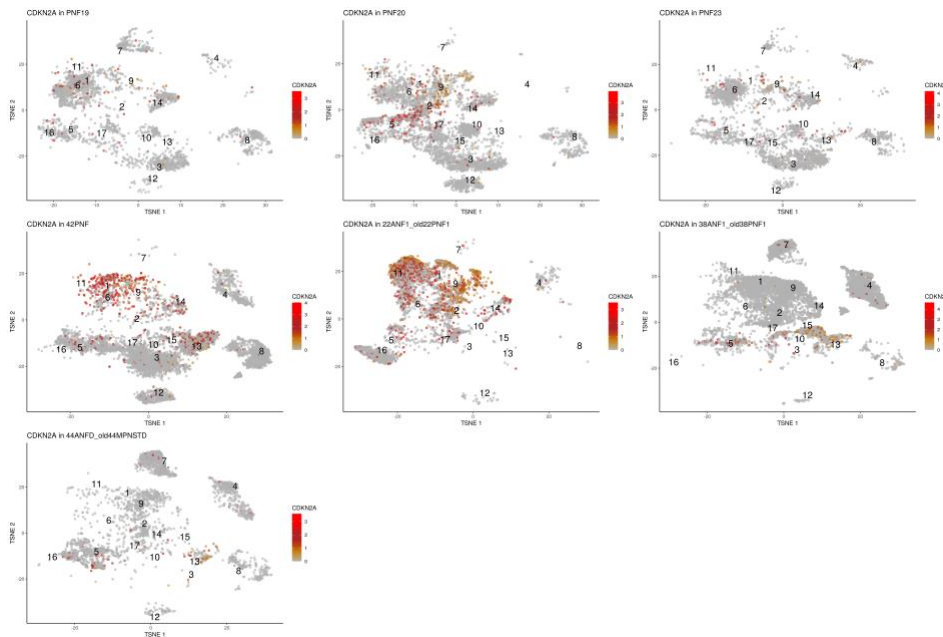


**Figure 11– Main cell components per sample (barplots).** Barplots showing the proportion of each cell type in each sample. **Note:** While iPS cells can not exist in a primary tissue like the ones studied here, they were identified probably due to the incompleteness of the reference datasets regarding our tissues of interest, which we expect to partially improve with the execution of this project.

## C – Differences in gene expression in the different samples

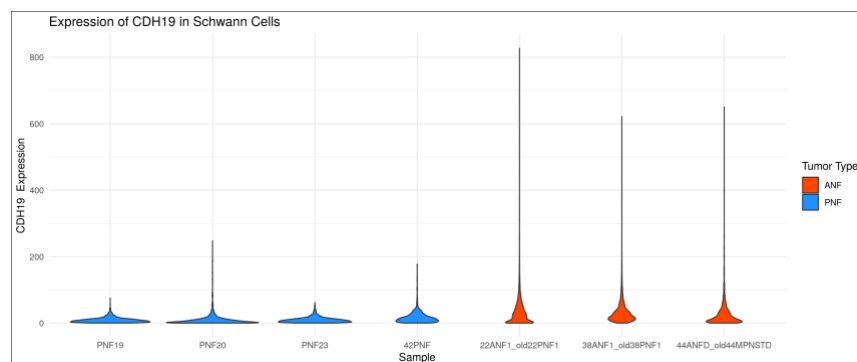
In addition to differences in the cellular composition of the different samples, we can observe differences in the expression of some important genes.

One example is *CDKN2A*. Its inactivation defines at the molecular level the transition from PNF to ANF and so we expect its expression to be decreased in ANFs (Figure 12). While the differences are not clear cut, we can see how in 38ANF and 44ANF the expression of *CDKN2A* in Schwann cells is almost null as expected. In the case of 22ANF, which presents a very high expression level of *CDKN2A*, the inactivation of this gene is caused by a translocation event. We hypothesize that it might be expressed in a non-functional form and its absence activates a feedback loop that increases its expression to levels even higher than those seen in PNFs.



**Figure 12 – *CDKN2A* expression per sample.** The expression level of *CDKN2A* in the different samples show slight differences between samples and tumor types.

Similar differences can be seen in other genes. For example, while the number of cell expressing *CDH19* does not change between PNFs and ANFs, the actual expression level is somewhat higher in ANFs (Figure 13).



**Figure 13– *CDH19* expression in Schwann cells.** Violin plots representing the expression levels of *CDH19* in all cells identified as Schwann cells in the different samples. A clear difference between PNFs (blue) and ANFs (red) can be seen.

What opportunities for training and professional development has the project provided

**Training:** One-on-one work with a mentor. Pere Pericot was an undergraduate student in our lab for 6 months under the supervision of Dr. Bernat Gel. He worked in the area of single cell data analysis of NF1 related tumors. He presented a poster at the Children's Tumor Foundation Conference "NF conference 2022" (see **Appendices**).

How were the results disseminated to communities of interest?

**Nothing to Report**

## **4. IMPACT**

### **Nothing to Report**

## **5. CHANGES/PROBLEMS**

There have been two changes in the execution of the present project:

### 1) Small delay in obtaining the RNA-seq data from all proposed samples.

During these first 12 month of the project, we established obtaining the RNA-seq data by 10x Genomics and SMART-seq analyses of the 16 samples proposed. However, given the value of the samples and the different conditions needed to set up for each of the different groups of samples (nerves, neurofibromas, MPNSTs), we prioritized first to set up the whole single cell analysis workflow (sample dissociation; single cell preparation; library constructions; sequencing; bioinformatic analysis) of few samples of the different tumor/tissue types, instead of performing sample preparation and sequencing for all 16 samples, before checking that the whole workflow was performing as expected. Since we know now that the workflow has been set up for all different types of tissue/tumors, now we can proceed with the analysis of all samples. This change in the order of established priorities (that is, finish the whole workflow up to sequencing analysis for a few samples of each type instead of performing the sequencing of the 16 samples before performing a bioinformatic quality control of the data) delayed just a bit the completion of the RNA-seq analysis proposed. However, this is just a small delay, since at the end of the summer, all 16 samples will be already completed and we will catch up time, since the bioinformatic pipelines are already ready to be executed for all of them.

### 2) Adding non-planned single cell data to the dataset

One of the aims of the present project is to generate a shared resource consisting in a comprehensive cell diversity map of the cellular composition, spatial distribution and genomic content of NF1-associated peripheral nerve sheath tumors. In order to make this single cell data set even more complete, we decided to include single cell epigenetic information, initially not planned to be part of this data set, that has been generated in the context of another previous project. In this regard, with no additional cost for the present project, there will be another layer of information (ATAC-seq data) from the same samples, in the final repository.

## 6. PRODUCTS

Publications, conferences, papers and presentations:

**Poster:** “Single-cell analysis of Plexiform Neurofibromas: heterogeneity and tumor cell-of-origin”

Pere Pericot, Miriam Magallon-Lorenz, Helena Mazuelas, Itziar Uriarte, Holger Heyn, Meritxell Carrió, Eduard Serra & Bernat Gel

Children’s Tumor Foundation **2022 NF Conference**, Philadelphia June 18-21 2022

**Poster:** “A genomic portrait of MPNST cells: governed by genomic structural and copy-number alterations and a mesenchymal identity”

Miriam Magallon-Lorenz, Ernest Terribes, Helena Mazuelas, Sara Ortega, Edgar Creus, Ignacio Blanco, Héctor Salvador, Nancy Ratner, Margaret Wallace, Juani Fernández-Rodríguez, Conxi Lázaro, Meritxell Carrió, Eduard Serra & Bernat Gel

Children’s Tumor Foundation **2022 NF Conference**, Philadelphia June 18-21 2022

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked in the project?

Name	Helena Mazuelas
Project Role	Postdoctoral Fellow
Researcher Identifier	0000-0001-7212-2144
Nearest person month worked	12
Contribution to the project	Dr. Mazuelas worked in the area of setting up conditions and preparing samples for single cell analysis

Name	Meritxell Carrió
Project Role	Wet Lab coordinator
Researcher Identifier	0000-0002-1258-6593
Nearest person month worked	2.4
Contribution to the project	Dr. Carrió coordinated and supervised the area of setting up conditions and preparing samples for single cell analysis

Name	Bernat Gel
Project Role	Dry Lab coordinator
Researcher Identifier	0000-0001-8878-349X
Nearest person month worked	2.4
Contribution to the project	Dr. Gel set up bioinformatic tools for single cell data analysis

Name	Eduard Serra
Project Role	PI. Project coordinator
Researcher Identifier	0000-0003-2895-9857
Nearest person month worked	2.4
Contribution to the project	Dr. Serra supervised and directed the project

Have there been a change in the active other support of the PI?

**Nothing to report**

What other organizations were involved as partners?

**Organization Name:** Centro Nacional de Análisis Genómico (CNAG-CRG);

**Location of organization:** Barcelona, Spain

**Partner's contribution to the project:** Single Cell Genomic group at the CNAG-CRG; **Facility:** Single Cell genomic facility; **Collaboration:** Dr. Holger Heyn Lab

## **8. SPECIAL REPORTING REQUIREMENTS**

**Nothing to report**

Single-cell analysis of Plexiform Neurofibromas: Heterogeneity and tumor cell-of-origin

Pier Perroti<sup>1</sup>, Miriam Magallon-Lorenz<sup>1</sup>, Helena Maziulis<sup>1</sup>, Izhar Uriarte<sup>1</sup>, Helger Heyn<sup>2</sup>, Meritxell Carró<sup>1</sup>, Eduard Serra<sup>3</sup> & Benoit Gail<sup>1</sup>  
 1) Hereditary Cancer Group, Genmasa Tissue Pathology Research Institute (GTP), Can Puig Campús, Barcelona, Barcelona, 08916, Spain; CIBERONC  
 2) Centre for Genomic Regulation (CRG-CRG), Barcelona Institute of Science and Technology (BIST), Barcelona, Spain  
 3) Centre for Genomic Regulation (CRG-CRG), Barcelona Institute of Science and Technology (BIST), Barcelona, Spain

Background

Plexiform neurofibromas (pNFs) are large and complex tumors that grow along large nerves and can compress vital structures. These benign tumors can potentially undergo malignant progression towards an MPNST, which makes them key contributors to reduced life expectancy in patients with NF1.

NF1(+), Schwann cells and NF1(+/-) endoneurial fibroblasts are the main cell types present in these tumors, although peripheral cells, including immune cells, stromal and others are also found within them. The precise identity of the cell-of-origin of pNFs remains a matter of discussion, as well as which are the mechanisms that lie behind the process of malignantization of pNFs towards MPNSTs.

With new emerging genomic and bioinformatics technologies, such as single-cell genomics and epigenomics, many different possibilities to interrogate these tumors arise. However, we are still missing a profound dissection of this kind of data allowing for a better understanding of the underlying biological structure of these tumors.

Objectives

This project aims to achieve the following objectives:  
 1) To study and characterize the cellular diversity that exists within plexiform neurofibromas, as well as to determine the heterogeneity of Schwann cells that compose them.  
 2) To determine if there exist NF1(-) cells within pNFs with the potential to differentiate towards a mesenchymal phenotype, the existence of which would point towards them potentially being the cells originating MPNSTs.  
 3) To assess whether this same diversity is observed in pNFs across in vitro 3D neurofibroma models not.

Methods

We performed an in-depth genomic and bioinformatic analysis of 3 pNFs, which were obtained in a recent study in our lab (Maziulis et al. 2022).

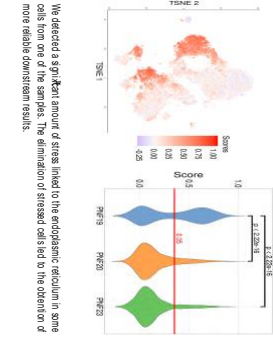
In order to characterize the whole transcriptome and genome accessibility profiles at single-cell resolution, we combined the analysis of standard scRNA-seq data along with the multiome technology by 10x Genomics, which allows us the generation of both single-cell RNA-seq and single-cell gene expression libraries from the same starting material.

Independently, we performed a more genetic analysis of scRNA-seq data using iPSC-based 3D neurofibroma models recently generated in the lab (Maziulis et al. 2022).

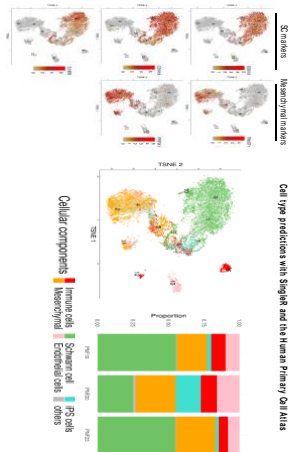
Bibliography

Maziulis et al. (2022) Cell Reports DOI: 10.1016/j.celrep.2022.110355

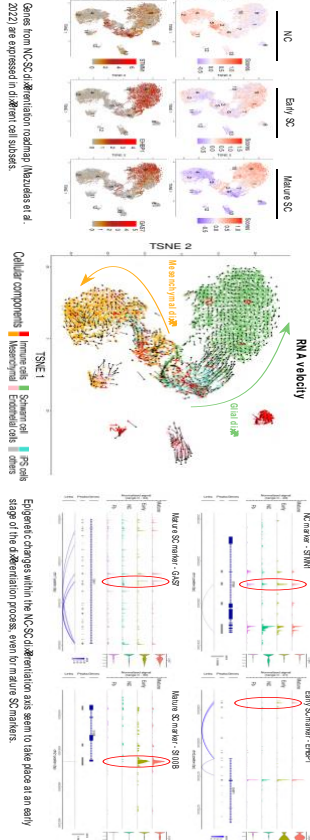
1 Correction of cellular artifacts among samples is essential for proper downstream analysis



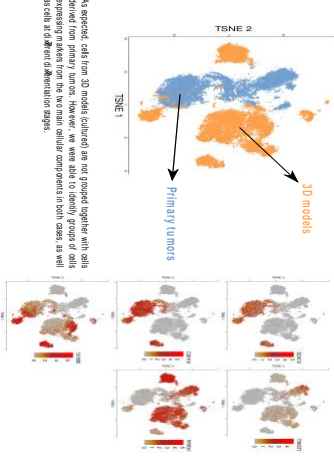
2 We identify the expected cellular components along with their expected proportions



3 Evaluation of diversity within the SC component reveals the presence of cells at diverse stages of differentiation within the NC-SC axis, which is driven by transcriptional and epigenetic changes



4 iPSC-based in vitro 3D neurofibroma models mostly recapitulate the cellular composition of primary tumors



Conclusions

- We observe cellular diversity within pNFs, and we find the expected components and proportions.
- The heterogeneity within SCs is partially due to the presence of cells at different stages of the NC-SC differentiation axis.
- NC-SC differentiation implies transcriptional and epigenetic changes, which appear to be decoupled.
- iPSC-based in vitro 3D neurofibroma models seem to recapitulate the cellular composition of primary tumors.
- Quality control procedures that go beyond the correction of artifacts that appear during library generation and sequencing are essential.

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FUNDACION PROYECTO GENOMICO  
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 Departament de Recerca i Innovació Tecnològica  
 Departament de Salut

La Marató

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