AWARD NUMBER: W81XWH-21-1-0483

TITLE: NF1-Associated Peripheral Nerve Sheath Tumors at Single-Cell Resolution: Heterogeneity, Tumor Growth, and Malignant Progression

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REPORT DATE: July 2023

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE:**	**3. DATES COVERED**
July 2023	**Annual**	**25Jun2022 – 24Jun2023**

4. TITLE AND SUBTITLE

NF1-Associated Peripheral Nerve Sheath Tumors at Single-Cell Resolution: Heterogeneity, Tumor Growth, and Malignant Progression

5a. CONTRACT NUMBER
W81XWH-21-1-0483

5b. GRANT NUMBER
NF200051

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER
0011613883

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fundacio Institut d'Investigacio En Ciencies de la Salut Germans Trias i Pujol
Carretera de Can Ruti. Camí de les Escoles s/n
08916 Badalona (Barcelona) Spain

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

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 subpopulations, not solely in the overall tumor.

15. SUBJECT TERMS None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRDC
a. REPORT	b. ABSTRACT	c. THIS PAGE	Unclassified	32	19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified			

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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 1-24:

As stated in the SOW

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	**Timeline (Months)**	**Site 1 GSU**
Major Task 1: 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
Subtask 8: Spatial Transcriptomics Samples used: Same samples used for Subtask 1 At least 4 samples of each. R1, PG, HH	8-24	X
Subtask 8: Spatial Transcriptomics bioinformatic analysis; PG, HH	18-30	X
Milestone(s) Achieved: Expression data and analysis from bulk RNAseq,	18	

scRNAseq, Smart- seq. Upload raw data and bioinformatic analysis to Synapse site.		
Major Task 2: Genetic and genomic analysis		
Subtask 1: Mutation analysis from Smart-seq data; R2	12-18	X
Subtask 2: Mutation analysis in specific subpopulations; R2	18-26	X
Subtask 3: MissionBio DNA+ targeted multi-omics; HH	6-24	X
Subtask 4: MissionBio DNA+ targetes mulit-omics bioinformatic analysis	18-30	X
Specific Aim 2: To elucidate the role in pNF growth of the different subpopulations of cells conforming the Schwann cell (SC) and the endoneurial fibroblast-like stromal (FB) components (12-30 months)		
Major Task 1: Separation of cell subpopulations within pNF SC and pNF FB components		
Subtask 1: Selection of cluster-specific markers based on Specific Aim 1 results	12-24	X
Subtask 2: Tumor dissociation and cell subpopulation separation. Samples used: Human pNF samples used for Subtask 1 Aim1. R2 At least 4 pNFs used	12-24	X
Milestone(s) Achieved: Conditions for the separation of different cell subpopulation established	24	
Major Task 2: SC and FB Subpopulation functional analysis		
Subtask 1: Analysis of potency and stemness of pNF SC subpopulations Samples used: At least 4 pNFs	18-30	X
Subtask 2: Analysis of niche effect and trophic capacity of FB subpopulations; R2 Samples used: At least 4 pNFs	18-30	X
Specific Aim 3: To identify and characterize the exact identity of the cell type within pNFs progressing towards aNF and MPNSTs and reproduce it in a human *NF1* (-/-) iPSC-based *in vitro*/*in vivo* 3D tumor model to analyze treatment responses		
Major Task 1: Identification of cells undergoing tumor progression using a *NF1*(-/-) iPSC-based 3D heterotypic spheroid model		
Subtask 1: Subpopulation-specific marker characterization in 3D heterotypic spheroids; R2 Samples used: 3 independent *NF1*(-/-) iPSC-derived neural crest cell lines, generated in our lab. Human eFBs derived from pNFs to generate heterotypic spheroids	12-24	X

Subtask 2: *In vitro* Selumetinib treatment in NF1(-/-) 3D heterotypic spheroids Samples used: 3 independent *NF1*(-/-) iPSC-derived neural crest cell lines generated in our lab. Human eFBs derived from pNFs to generate heterotypic spheroids	18-30	X
Major Task 2: Pilot *in vivo* Selumetinib treatment in neurofibroma-like model		
Subtask 1: Submit document for ACURO Approval	1-20	
Milestone(s) Achieved: Obtain ACURO approval	20	
Subtask 2: Generation of heterotypic spheroids Samples used: 3 independent *NF1*(-/-) iPSC-derived neural crest cell lines generated in our lab. Human eFBs derived from pNFs to generate heterotypic spheroids	22-24	X
Subtask 3: Generation of reporter spheroids Samples used: spheroids generated in Subtask 2. GFP and luciferase reporter lentivirus (ready to use viral stocks)	23-24	X

What has been accomplished

Thereafter it is described the work accomplished between months 13-24. It involves both, work specifically framed in this period of time in the SOW document, and also work that in the previous report was reported as ongoing and flagged as "partially achieved".

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	**Timeline (Months)**	**Site 1 GSU**
Major Task 1: Expression analysis: bulk RNAseq, scRNAseq, spatial transcriptomics		
Subtask 1: HRPO review and approval	1-4	
Achieved		
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
Achieved **Update at 24 months:** As mentioned in the previous report, we gave preference to aNF (ANNUBP) and nerves tissue, to single cell analysis. After performing scRNA-seq and SMART-seq we were able to perform bulk RNA-seq from 3 aNF and from 3 control nerves. A biological triplicate of these tissues will be sufficient to perform the comparative analysis with single cell expression analyses. However, as we are still prospectively collecting aNF and nerve samples, if we receive more samples in the coming months, we will perform additional bulk RNA-seq analysis of these samples. We already have the results from bulk RNA-seq analysis of all samples (see subtask 3)		
Subtask 3: Bulk RNAseq bioinformatic analysis	8-12	X
Achieved **Update at 24 months:** Bulk-RNA-seq analysis of all samples has been performed.		
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

Achieved **Update at 24 months:** We have performed scRNAseq from 4 samples of each tumor type: 4 pNFs, 4 aNFs and 4 MPNSTs. In the case of nerve samples, we first had to set up an *ad hoc* dissociation protocol for nerve tissue using a myelin removal step (see the previous 12-month report) which is mandatory because myelin inhibits cell lysis required for single cell analysis. However, during this process, lots of cells are lost during the myelin removal step, making this analysis really challenging. The Single Cell Unit at CNAG followed a low input specific protocol for this kind of samples and we have been able to already sequence 2 nerve samples after several attempts of library preparation. Following this success, a couple of more nerves have been prepared and are being single cell analyzed. We already have the results from the bulk-RNAseq analysis of all processed samples (see subtask 5).		
Subtask 5: scRNAseq bioinformatic analysis	8-12	X
Achieved scRNAseq analysis has been performed for 5 PNFs, 4 ANNUBPs, 4 MPNSTs, and 2 nerves (2 more when sequences will be delivered). A data analysis pipeline was put together, containing preprocessing and QC steps; stress detection and removal; batch effect correction; single cell level cell type identification. Additional analyses include cluster-specific marker gene identification and individual gene expression; differential expression analysis; cell differentiation/maturation status analysis; copy-number profiles; RNA-velocity, etc. Some examples of the single cell expression analysis can be found below in the "**Document on single cell expression analysis**". As mentioned in the previous report, scRNAseq data was obtained from the same set of cells as scATAC-seq data as part of a single-cell multiome analysis (scATAC-seq is supported by a different project but data will be deposited together with single cell data generated in this project).		
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
Partially achieved After establishing a protocol for FACS dissociated cell of each tumor/tissue type, Smart-seq has been achieved for the same tumor samples as in Subtask 4. Again, nerves samples were the most difficult to process, due to the presence of large amounts of myelin, a component that inhibits any enzymatic reaction, complicating the manipulation of single cells. For scRNA-seq (10x genomics) we have been able to process 2 nerves and 2 are currently being sequenced. However, for Smart-seq, we have been able to sequence 1 nerve, and are in the process of preparing libraries from		

additional nerves.		
Subtask 7: Smart-seq bioinformatic analysis	8-18	X
Partially achieved A pipeline analysis for Smart-seq data has been stablished. A delay in obtaining all sequences from the prepared libraries also affected the bioinformatic analyses. However, now, despite not having the complete set of nerve samples, data for all tumors is being analyzed and we expect to finish it in the following months		
Subtask 8: Spatial Transcriptomics Samples used: Same samples used for Subtask 1 At least 4 samples of each. R1, PG, HH	8-24	X
Partially achieved Spatial Transcriptomic analysis is being finished. It is performed on OCT-embedded samples from pNFs, aNFs, MPNST and nerves. This technique requires several steps: 1) Quality control (QC) of RNA from OCT-embedded tissue. This is critical, since low RNA quality will not permit continuing with the process. 2) Tissue Optimization conditions for all different tissues/tumor types need to be set up to establish the permeabilization time for each tissue type. 3) cDNA library preparation and sequencing. All our OCT-embedded samples passed the QC for RNA quality. Tissue optimization have been performed successfully for the 4 different tissue types. In a first experiment, we performed Spatial transcriptomic on 3 pNFs, 1aNFs (ANNUBP) and 4 MPNSTs. For these samples we have finalized the whole process and already obtained sequencing data. In a second experiment, we performed Spatial Transcriptomics on nerve tissue. We included 3 control nerves and 1 nerve within a pNF. For these samples we are waiting for the sequencing results. As we already stated in the project application, aNF (ANNUBP) samples are being prospectively collected. We prioritized tissue for single cell expression techniques. We are currently collecting more aNF samples for Spatial Transcriptomics. See below the document "**Spatial Transcriptomics Setup**" for an overview of the procedures performed.		
Subtask 8: Spatial Transcriptomics bioinformatic analysis; PG, HH	18-30	X
We have setup a basic analysis pipeline for SpatialTranscriptomics data including the initial preprocessing of the data with SpaceRanger and visualization with Loupe Browser, followed by additional QC and analysis with R/Bioconductor packages. The delay in data generation impacted the execution of this subtask. Therefore, we are still working on the advanced analyses and comparisons, including subspot resolution enhancement, spatially guided differential gene expression and specially spatially informed cell type deconvolution using the scRNAseq subpopulations as		

references.		
Milestone(s) Achieved: Expression data and analysis from bulk RNAseq, scRNAseq, Smart- seq. Upload raw data and bioinformatic analysis to Synapse site.	18	
Almost achieved With the exception of the single cell expression data for nerve samples (2 last nerves are being sequenced with 10x technology and Smart-seq libraries for 2 new nerves are ongoing) all bulk and single cell expression data has been generated. All these data are currently being uploaded into Synapse. All content already uploaded can be checked here: DOI: https://doi.org/10.7303/syn52146919		
Major Task 2: Genetic and genomic analysis		
Subtask 1: Mutation analysis from Smart-seq data; R2	12-18	X
Partially achieved We are in the process of implementing a nextflow-based data analysis pipeline for the identification of mutations in Smart-seq data taking advantage of the full transcriptome generated for each cell. Preliminary results showed promise in assigning mutational status to the different cellular subpopulations identified using 10X technologies. We expect to achieve this task in the next following months. As an orthogonal approach to assign a genetic and genomic alteration status to cells to complement mutational status from Smart-seq we have implemented an analysis for copy-number estimation from scRNA-seq data, which has proven useful for MPNSTs and are now testing for ANNUBPs (see document "**Copy-number estimation from scRNA-seq data**")		
Subtask 2: Mutation analysis in specific subpopulations; R2	18-26	X
Partially achieved This task has been impacted by the delay in Subtask 1 and the delay in cluster specific marker identification (see Specific Aim 2, Major Task 1, Subtask 1). We will use the same approach we are implementing for subtask 1 to the mutational analysis of specific subpopulations defined from 10X data		
Subtask 3: MissionBio DNA+ targeted multi-omics; HH	6-24	X
Pending We proposed the use of the platform Tapestry from MisionBio, for performing a single-cell multi-omics (DNA+protein), in order to identify specific genetic pathogenic variants (SNVs and CNVs) in specific cell subpopulations, characterized by the expression of specific proteins. In one		

hand, this technology is especially expensive and on the other, we were constrained by the multiplexed format (in sets of 8 samples). That is why we proposed to only analyze a biological triplicate of aNF (ANNUBPs) and MPNSTs, and two pNFs (8 samples in total), since aNFs and MPNSTs are the tumors exhibiting genetic alterations associated with tumor progression. In order to determine the specific cell population containing precise genetic alterations, we proposed to use 2-3 Ab for determining cell identity in pNFs and aNFs and 2-3 in MPNSTs. Given the high cost of this technique and the delay in identifying the right markers and Abs for the precise determination of cell identity for aNFs and MPNSTs (see below **Specific Aim 2, Major Task 1, Subtask 1**), we prefer to wait until we have a robust decision on the markers and Abs to use.		
Subtask 4: MissionBio DNA+ targetes mulit-omics bioinformatic analysis	18-30	X
Pending There is still no bioinformatic analysis, since data has not been generated yet.		
Specific Aim 2: To elucidate the role in pNF growth of the different subpopulations of cells conforming the Schwann cell (SC) and the endoneurial fibroblast-like stromal (FB) components (12-30 months)		
Major Task 1: Separation of cell subpopulations within pNF SC and pNF FB components		
Subtask 1: Selection of cluster-specific markers based on Specific Aim 1 results	12-24	X
Partially achieved This is a crucial task with implications in a number of other tasks including MissionBio data generation. While we originally proposed to select markers for clusters defined by scRNA-seq, since scATAC-seq data for the exact same cells is now available, we decided to define the cell subpopulations based not only on transcriptomic data but also on chromatin accessibility profile for a better and more reliable cell cluster definition. We have designed and implemented the data analysis for the integration of these data types and the definition of cell clusters. However, for the definitive cluster structure and the identification of subpopulation specific markers based on that, we need access to the whole dataset, which has been delayed due to the technical problems for nerve processing. Now that we have an almost complete 10X dataset we expect to be able to define the cell cluster structure and identify subpopulation markers in the next few months. Once we have these available, we will be able to proceed with all task depending on this one.		
Subtask 2: Tumor dissociation and cell subpopulation separation. Samples used: Human pNF samples used for Subtask 1 Aim1. R2	12-24	X

At least 4 pNFs used		
Partially achieved As a proof of concept, we have set up specific conditions to sort the Schwann cell (SC) fraction and the endoneurial fibroblasts from plexiform and atypical neurofibromas, using an already known marker. Since p75/CD271 is expressed in SCs but not in fibroblast, we used this cell membrane marker to separate p75 positive cells (glial cells) from the rest of cells (p75 negative, most being fibroblasts). We have established two different protocols: one based on Fluorescent Activated Cell Sorting (FACS) and another one based on Magnetic Activated Cell Sorting (MACS). For further information, please see below the document entitled "**aNF and pNF Cell separation**". Once marker and Abs are selected for separating specific subpopulations of pNF and ANNUBP cells, we will use the exact same set up conditions, but using specific dedicated Abs.		
Milestone(s) Achieved: Conditions for the separation of different cell subpopulation established	24	
Partially achieved We have set up a methodology for dissociating cells from pNFs and set up a proof of concept for cell isolation using p75/CD271 either by MACS or FACS. We are ready for doing the same with selected markers and Abs for the separation of specific SC subpopulations.		
Major Task 2: SC and FB Subpopulation functional analysis		
Subtask 1: Analysis of potency and stemness of pNF SC subpopulations Samples used: At least 4 pNFs	18-30	X
Pending		
Subtask 2: Analysis of niche effect and trophic capacity of FB subpopulations; R2 Samples used: At least 4 pNFs	18-30	X
Pending		
Specific Aim 3: To identify and characterize the exact identity of the cell type within pNFs progressing towards aNF and MPNSTs and reproduce it in a human *NF1* (-/-) iPSC-based *in vitro*/*in vivo* 3D tumor model to analyze treatment responses		
Major Task 1: Identification of cells undergoing tumor progression using a *NF1*(-/-) iPSC-based 3D heterotypic spheroid model		
Subtask 1: Subpopulation-specific marker characterization in 3D heterotypic spheroids; R2 Samples used: 3 independent *NF1*(-/-) iPSC-derived neural crest cell lines, generated in our lab. Human eFBs derived from pNFs to generate	12-24	X

heterotypic spheroids		
Partially achieved See above **Specific Aim 2, Major Task 1, Subtask 1**,		
Subtask 2: *In vitro* Selumetinib treatment in NF1(-/-) 3D heterotypic spheroids Samples used: 3 independent *NF1*(-/-) iPSC-derived neural crest cell lines generated in our lab. Human eFBs derived from pNFs to generate heterotypic spheroids	18-30	X
Partially achieved We have successfully generated neurofibromaspheres derived from NF1 (-/-) iPSCs and pNF-derived primary fibroblasts, and treated them with Selumetinib, as a proof of concept. For further details, please see below "**In vitro Selumetinib treatment in NF1(-/-) heterotypic spheroids**". In our lab, we have generated additional iPSCs bearing the inactivation of additional tumor suppressor genes: CDKN2A and SUZ12 (a component of the PRC2). Spheres representing aNFs and MPNSTs are also regularly being generated and are ready for the use in the present project. We are ready to assess the impact of Selumetinib on specific cell populations, in all spheroid-based models. As soon as markers and Abs are chosen, experiments can be performed.		
Major Task 2: Pilot *in vivo* Selumetinib treatment in neurofibroma-like model		
Subtask 1: Submit document for ACURO Approval	1-20	
Pending As stated in the project, all animal experiments will be conducted in the animal facility of IDIBELL Research Institute, in collaboration with the group of Dr. Conxi Lázaro. The protocol for performing the proposed animal experiments is already developed and approved at IDIBELL by their IACUC, and is being currently used for other projects using identical animal procedures. We obtained this protocol from IDIBELL and the IACUC-approved decision on the use of the protocol. We are finishing the completion of the current version of the ACURO Animal Use Appendix and will submit everything via USArmy.detrick.medcom-usamrmc.other.acuro@mail.mil		
Milestone(s) Achieved: Obtain ACURO approval	20	
Pending Paperwork for obtaining ACURO approval are currently being processed.		
Subtask 2: Generation of heterotypic spheroids Samples used: 3 independent *NF1*(-/-) iPSC-derived neural crest cell lines generated in our lab. Human eFBs derived from pNFs to generate heterotypic spheroids	22-24	X

Partially achieved Heterotypic spheroids are regularly being produced in our lab (see Mazuelas et al. 2023; DOI:10.1016/j.xpro.2023.102198). For an example see the document "**In vitro Selumetinib treatment in NF1(-/-) heterotypic spheroids**"		
Subtask 3: Generation of reporter spheroids Samples used: spheroids generated in Subtask 2. GFP and luciferase reporter lentivirus (ready to use viral stocks)	23-24	X
Pending Viral have been for reporter generation have been purchased and are ready to use.		

Document on single cell expression analysis

scRNAseq

scRNAseq has been performed for 5 PNFs, 4 ANNUBPs and 4 MPNSTs and 2 nerves. ScRNAseq data call obtained from the same set of cells as scATAC data as part of a single-cell multiome analysis (as reported in the previous report).

Data for all samples after initial quality control and cleaning showed the expected batch (sample) effects, were cells from the same samples showed tendency to cluster together in the dimensionality reduction plots (**Figure 1**).

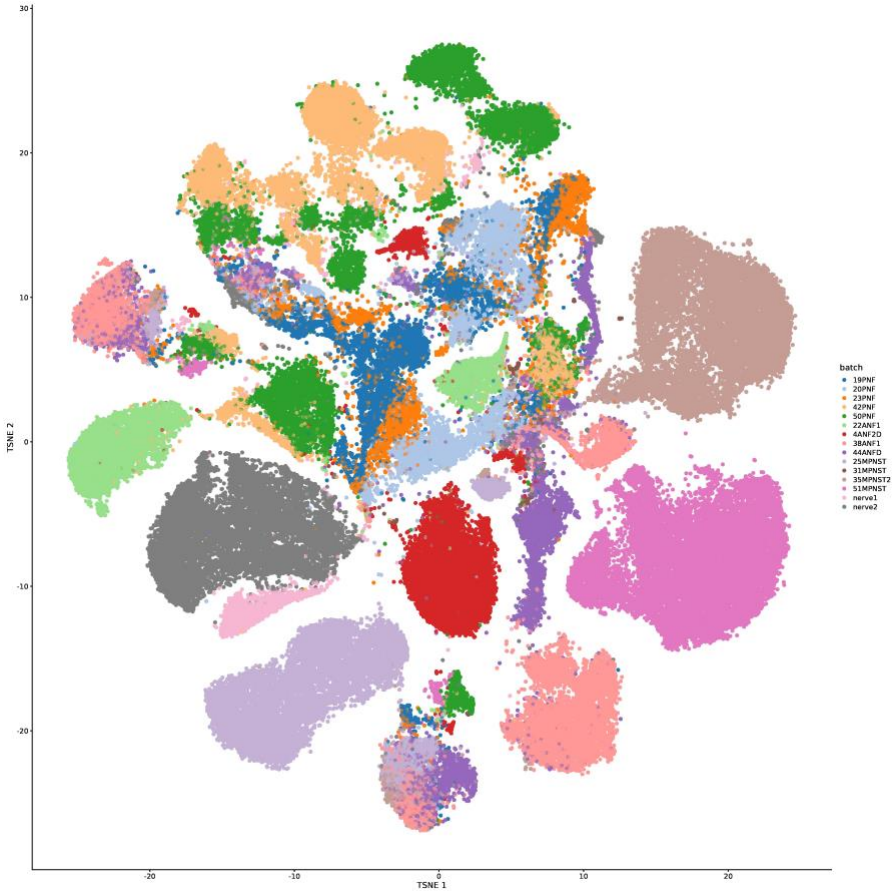


Figure 1 – *TSNE plot of scRNAseq data for all samples after an initial standard quality control. Each color represents a different sample.*

We performed an extended quality control and cleaning process to remove suspected doublets and highly stressed cells from each sample. This analysis revealed that some samples had a significant number of stressed cells (**Figure 2**). From our previous work we know that stressed cells can have an important influence on scRNAseq analysis and so we opted for removal of the stressed cells from the dataset.

After the removal of stressed cells, we performed a normalization process to remove the batch effects and make the different samples comparable. This process, as expected, resulted in a much more compact representation, with a clearer structure.

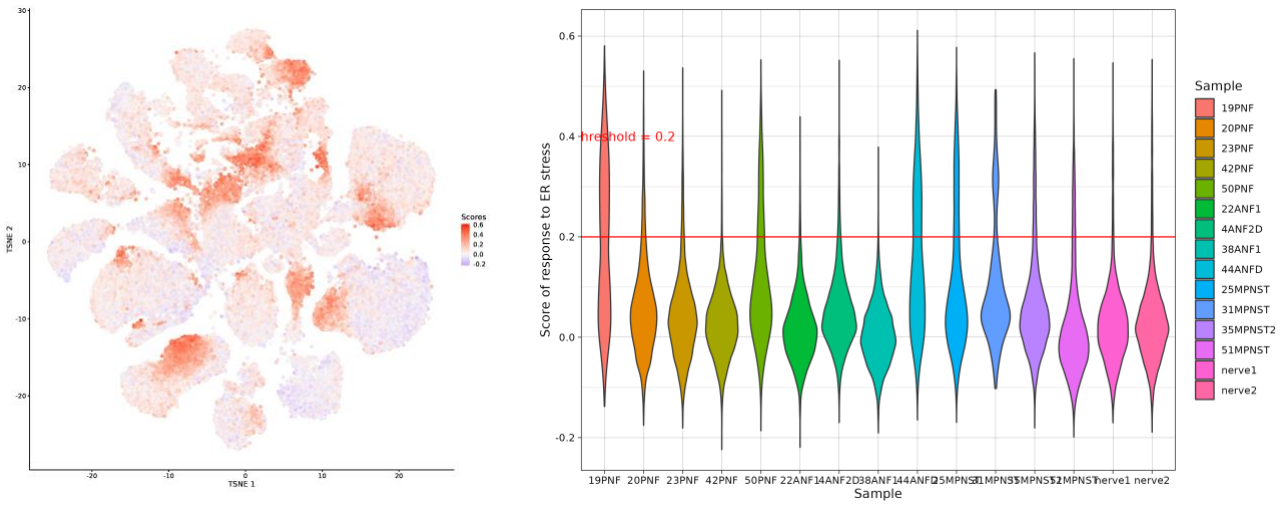


Figure 2 – *(left) TSNE plot of scRNAseq data for all samples with stress scores per cell. Red for high levels of stress. (right) Violin plot showing the distribution of stress levels per sample*

We identified cell clusters jointly in the multidimensional space and used a reference human cell atlas to assign cell types to the different cell clusters. The resulting TSNE plot showed a big central blob with glial and mesenchymal cells and independent small clusters corresponding to endothelial cells and immune infiltrate, including T-cells, B-cells and macrophages (**Figure 3**).

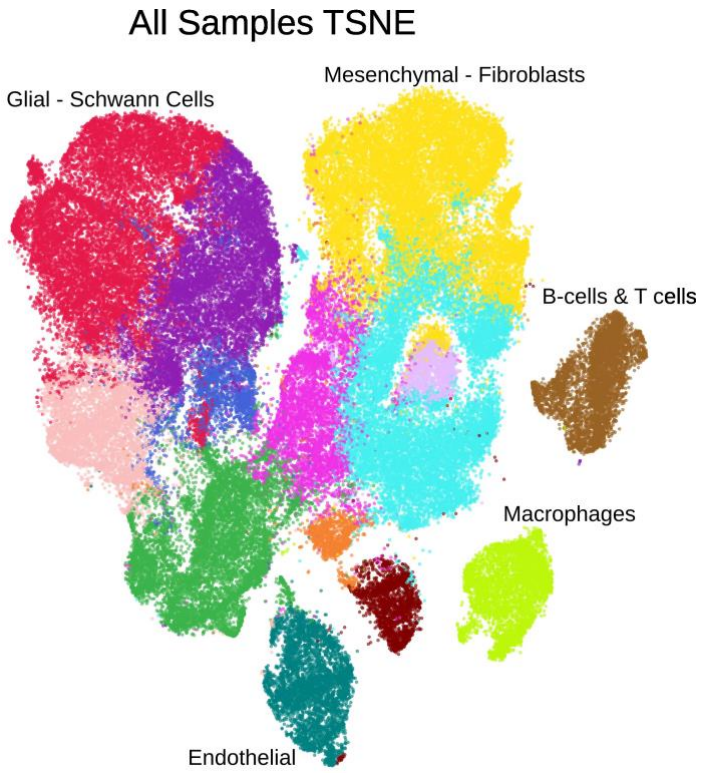


Figure 3 – *TSNE plot of all samples with cell types assigned.*

We are currently performing an exhaustive histological characterization of these samples to validate the proportions of the different cell types assigned. However, the *in-silico* cell type assignment matched with known cell type markers and clearly separated the main central blob into two different parts, the mesenchymal (Fibroblasts) and the glial (Schwann cells) part. Both parts are connected at the region that contain the less differentiated cells, which is concordant with our prior analyses (**Figure 4,** and see previous 12 month report).

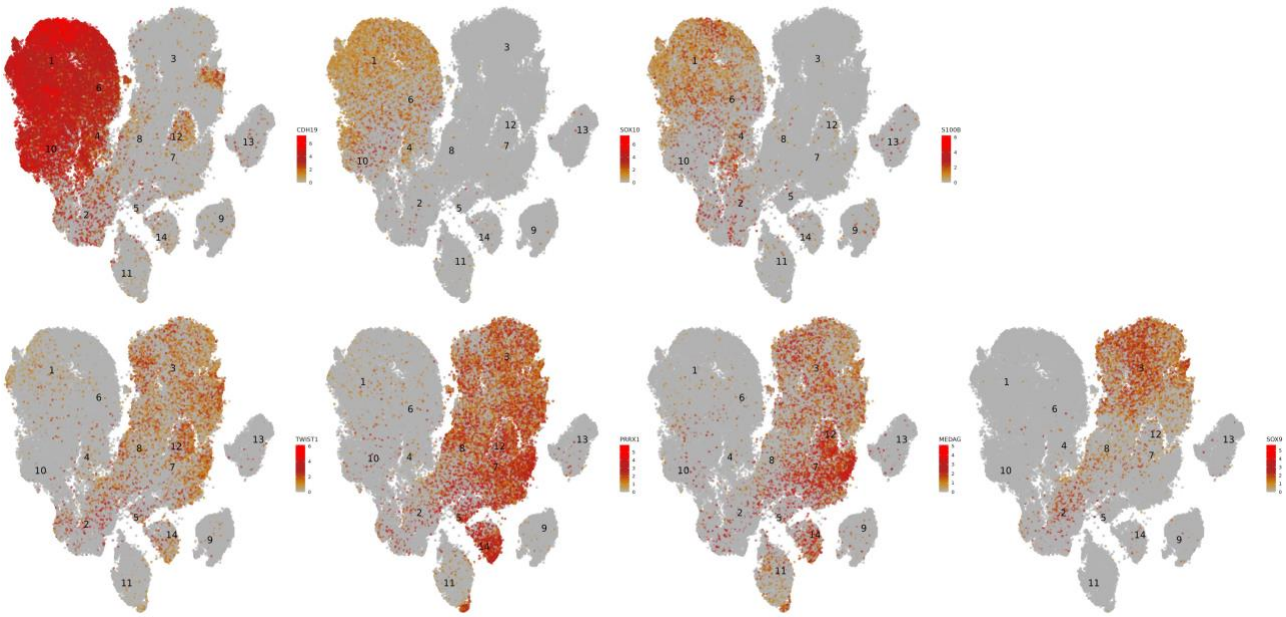


Figure 4 – *Known marker genes for Schwann cells (top row, left to right: CDH19, SOX10, S100B) and for mesenchymal – fibroblasts component (bottom row, left to right: TWIST1, PRRX1, MEDAG, SOX9).*

When separating this data per sample, we detected that one sample, 31MPNST, had lower number of cells than expected and, according to the initial quality controls, it might be due to a problem with sample processing. We are in the process of investigating the cause of this problem and exploring if it would be possible to correct it bioinformatically or we will need to process and sequence the sample again.

Below (**Figure 5**), we can observe the proportion of cell types per sample. We can appreciate the clear differences between sample types, including the expected mesenchymal identity of MPNST cells but also interesting differences in the glial component between PNF and ANNUBPs. We can also observe differences among samples within tumor types. Nerve tissue contains most of the cell types and identities present in pNFs and ANNUBPs, but not the distinctive mesenchymal-like cells present in MPNSTs, also absent in pNFs
and ANNUBPs.

Figure 5 – *Single cell RNAseq data from the different samples, from control nerves at the top to MPNSTs at the bottom. Each square corresponds to a single sample.*

Spatial Transcriptomics Setup

STEP 1. Quality control of RNA from OCT-embedded tissue samples

All samples had a RIN higher than 5.5 and could be included in the analysis.

Subproject	Researcher	Date at CNA	Librarie barc	Name of San	Species	QC H&E	QC RIN
SERRA_31	Edurard Serra Meritxell Carrió						
Cancer hereditary group		20/12/22	AZ8101	50PNF	Human	Yes	5,9
		20/12/22	AZ8102	44MPNSTE	Human	Yes	5,9
		20/12/22	AZ8103	42PNF Rosat	Human	Yes	5,7
		12/4/23	AZ8104	53 PNF	Human	Yes	7,3
SERRA_30		20/12/22	AZ8097	35MPNST2	Human	Yes	9
OCT tumor tissue/OCT nerve tissue		20/12/22	AZ8098	51MPNST	Human	Yes	6,5
		12/4/23	AZ8099	51 MPNST2	Human	Yes	8,3
		12/4/23	AZ8100	54 MPNSTB	Human	Yes	5,8

STEP 2. Tissue optimization conditions

Permeabilization time was set up independently for each different tissue (nerve, pNFs, sNFs and MPNSTs). Set permeabilization times are as follow:

- **pNFs and aNFs**: 6 minutes of permeabilization
- **MPNSTs**: 9 minutes of permeabilization
- **Nerves**: 12 minutes of permeabilization

STEP 3. Gene expression: cDNA library preparation and sequencing
We selected areas of 6,5mmx6,5mm of tissues to be analyzed. Each slide contains 4 spots for 4 different samples. **Figure 1** shows the distribution and selected tissue areas of the two slides performed. Note that for the first slide, 44MPNSTE is an aNF (ANNUBP) and not and MPNST.

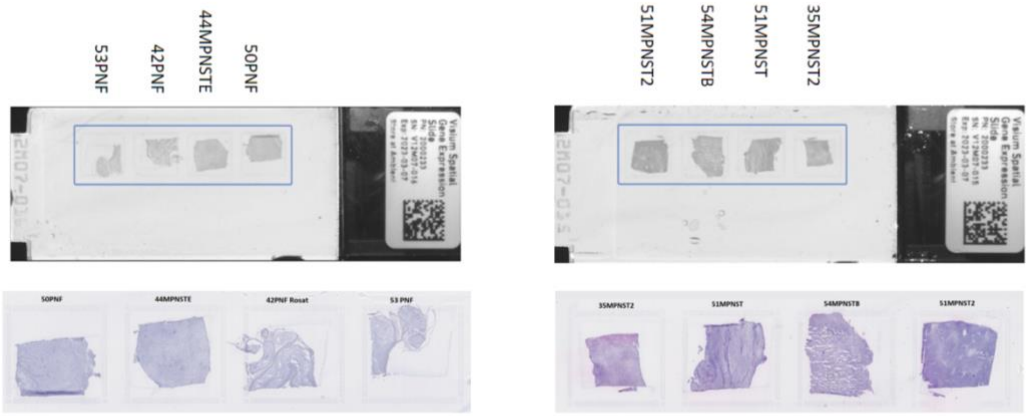


Figure 1. Selected areas of each sample tissue for composing two Spatial Transcriptomic slides (n= 8 samples).

Copy-number estimation from scRNA-seq data

With sufficient high quality data it is possible to estimate copy-number alterations from scRNA-seq transcriptomic data. The idea is that genomic gains and losses will have an impact on mRNA abundance that we can detect. This approach is based in very noisy data since the impact of copy number alterations on gene expression is very gene-specific. However, taking advantage of cell populations it is possible to obtain a good estimation of broad changes that are present in multiple cells.

We implemented such an approach and tested on MPNST data. **Figure 1** shows the results for one of the MPNSTs. We can see a portion of the cells with no alterations (green left bar) corresponding to the stromal component of the MPNST and a majority of MPNST cells (red left bar). MPNST cells share most alterations but not all of them, and we can see 2 main subpopulations with additional substruture on each of them. Based on this copy-number estimations we can identify the diploid/aneuploid cells in the different scRNA-seq defined clusters and even project the different subpopulations defined by genomics and explore their transcriptomic impact.

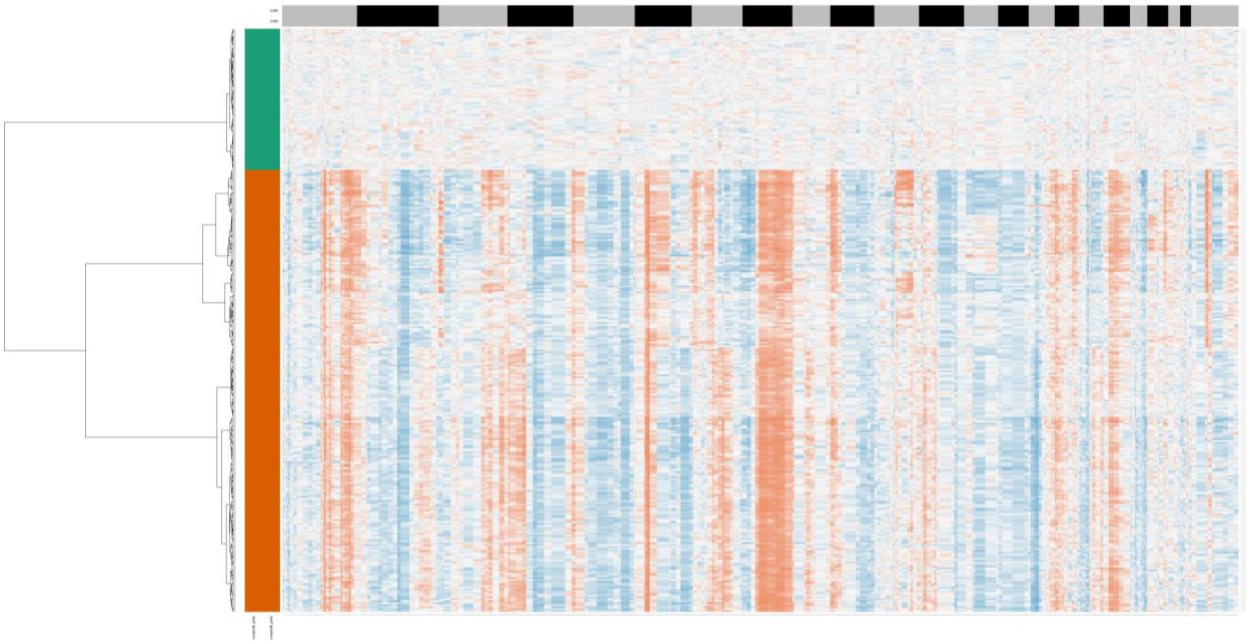


Figure 1 – *Heatmap depicting the single cell level copy-number estimation based on scRNA-seq data. Each row is an individual cells while the x-axis represents the genome, with alternating gray-black boxes on the upper part represent the chromosomes. Red stands for gains and blue for losses, while white corresponds to 2n.*

pNF and aNF Cell Separation

Procedure for separating the SC component and the fibroblast c omponent

We first dissociate plexiform or atypical neurofibromas in sterile conditions cutting a section of a tumor (either from fresh tissue or from cryopreserved tissue for culturing purposes) into very small pieces (approximately 2 mm x 2 mm) using scalpels. Afterwards, small pieces are submerged in dissociation media (DMEM + 10% FBS + P/S + Glutamax + Normocin + Colagenase + Dispase) and incubated at 37ºC and 5% CO2 for 16 hours. Next day, we mechanically dissociate the small neurofibroma pieces using a glass Pasteur pipette until a homogeneous cell suspension is obtained. Cell suspension is centrifuged twice to obtain a cell pellet which can be counted. Then, to cell sort the SC fraction, the cell suspension can be either labelled with p75 antibody (Cat# ab3125) (with the consecutive secondary antibody incubation) to perform **FACS** or labelled with the human CD271 Microbead kit from Milteny Biotec magnetic beads (Cat# 130-099-023) to perform **MACS (**see **Figure 1)**. Once the SC positive fraction is obtained either by FACS or by MACS, it is seeded in SC media in poly L-lysine and laminin-coated plates at 37ºC and 10% CO2. The negative fraction, mostly composed by fibroblast, can be seeded in DMEM + 10% FBS + P/S + Glutamax at 37ºC and 5% CO2.

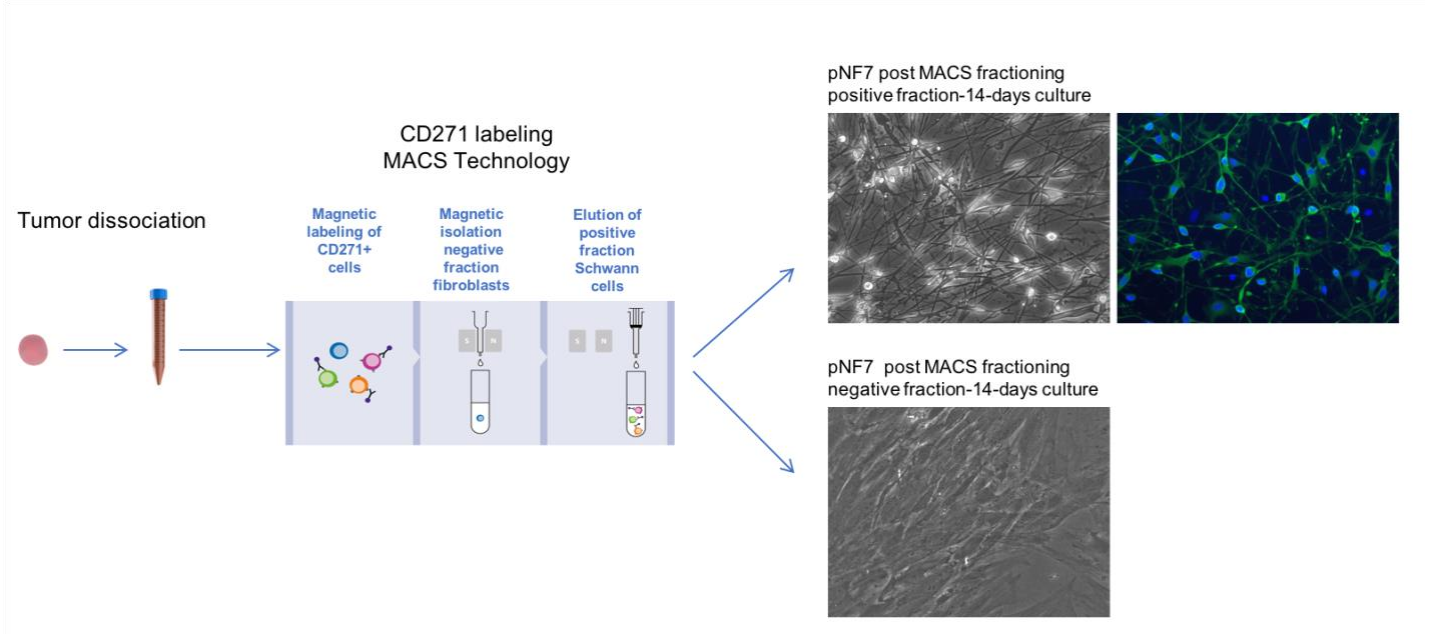


Figure 1. MACS-based separation of pNFs and cNFs SC and fibroblast component. After tumor dissociation, cell components are separated using Ab-conjugated magnetic beads. The negative fraction is basically enriched in tumor fibroblasts (among a minority of other cell types, like endothelial cells or immune cells) and the eluted positive fraction is enriched in SCs.

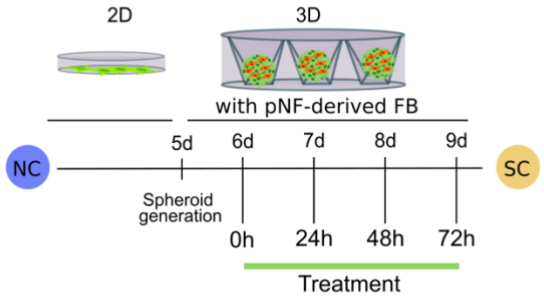
***In vitro* Selumetinib treatment in *NF1*(-/-) heterotypic spheroids**

We have set up assays to evaluate the treatment of Selumetinib in the neurofibromasphere model we have developed (Mazuelas et al. 2022, DOI: /10.1016/j.celrep.2022.110385).

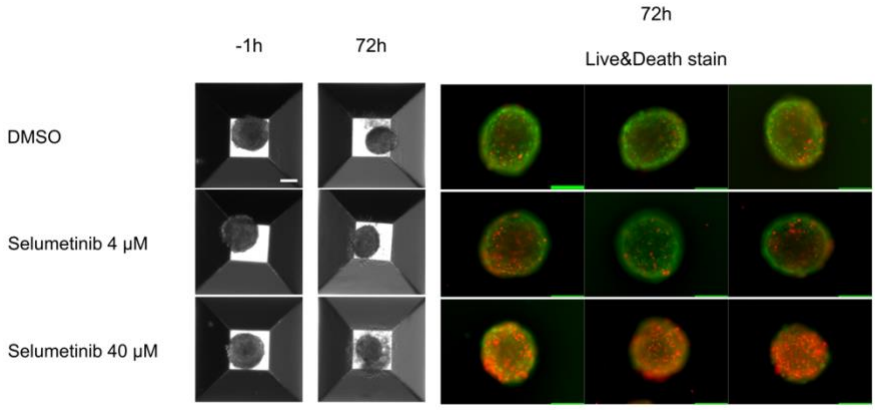
Experimental design

We generated neurofibromaspheres as shown in **Figure 1**. Briefly, we initiate Schwann cell differentiation from *NF1*(-/-) Neural Crest cells in 2D, and at day 5 we assemble neurofibromaspheres consisting of *NF1*(-/-) differentiating SC cells with *NF1*(+/-) fibroblasts isolated from neurofibromas. 24 hours after Neurofibromasphere formation, Selumetinib treatment is started that last for 72 hours.
Figure 1. Scheme of neurofibromaspheres generation and Selumetinib tretament

Determining Selumetinib concentration



After 72 hours of Selumetinib treatment, we perform live and death staining to visualize treatment response. As shown in **Figure 2** control spheroids treated with DMSO show compact morphology and only a few death cells, whereas treatment with Selumetinib at 4µM and 40µM doses showed disaggregation and either an increased or a high percentage of dead cells, respectively. Based on these



results we decided to use Selumetinib at 40µM for future experiments using neurofibromaspheres
Figure 2. Live (Acridine orange, green) and death (Propidium iodide, red) staining (AOI staining kit) showing the effect of 4µM and 40µM Selumetinib treatment.

Determining the number of cells per neurofibromasphere
We have also set up a Cell Viability Assay using the Cell Titer Glo 3D assay (an ATP luminescent-based cell viability assay commonly used for 3D microtissue spheroids). Neurofibromaspheres are generated and treated with Selumetinib in 96-spheroid plates from corning, as explained in **Figure 1**. Luminescence is measured after 72 hours of treatment. In a first set of experiments, we tested the effect of Selumentinib at 40µM concentrations on 3,000 and 5,000-cell spheroids. As shown in **Figure 3** we did not detect differences in Selumetinib treatment between different spheroid sizes, and we decided to use 5,000-cell spheroids for future experiments.

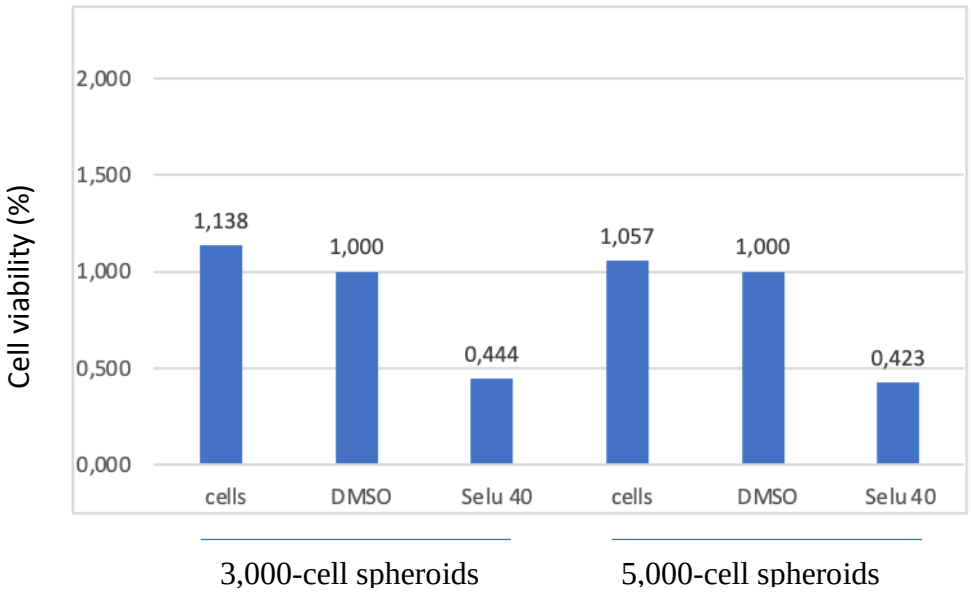


Figure 3. Effect of Selumetinib treatment on cell viability in neurofibromaspheres. Spheroids were generated and Selumetinib was added 24 hours later. Cell viability was measured using the Cell titer GLo 3D assay. The mean from at least 8 spheroids per condition is represented. Data are expressed as relative viability to DMSO-treated control cells.

4. IMPACT
Nothing to report.

5. CHANGES/PROBLEMS

The project is advancing at a good rhythm and the progress made is considerable. Most of the pending or on-going tasks mentioned in the previous report are at this point already achieved. However, despite this progress, we report here some delays, that are somehow related, in the consecution of the proposed work. Significantly, there are three delays that is worth to mention:

1) Delay in the obtention of single cell expression data from nerves
As we describe in section 3 “Accomplishments”, one of the problems that we faced with the different single cell technologies employed in this project has been the enzymatic high inhibitory capacity of myelin. None of the peripheral nervous systems studied (pNF, aNF, MPNST) contain myelin, so the only tissue that has been clearly problematic for us has been the nerves. Dissociation of nerve tissue, sample cleaning, separation of single cells, cell lysis and library preparation, etc, in any single step remaining myelin has been a problem. We have been able to improve and solve many of the steps and are now in a position of finishing the analysis in the following months. However, this caused a certain delay in obtaining all single cell data and more importantly, in the integration of all different types of analyses for the whole set of samples. As explained, we are now in a position to finish the analysis of nerves.

2) Delay in the marker identification for specific cell population separation
As reported, this is a crucial task with implications in a number of other tasks including MissionBio data generation. While we originally proposed to select markers for clusters defined by scRNA-seq, since scATAC-seq data for the exact same cells is now available, we decided to define the cell subpopulations based not only on transcriptomic data but also on chromatin accessibility profile for a better and more reliable cell cluster definition. We have designed and implemented the data analysis for the integration of these data types and the definition of cell clusters. However, for the definitive cluster structure and the identification of subpopulation specific markers based on that, we need access to the whole dataset, which has been delayed due to the technical problems for nerve processing. Now that we have an almost complete 10X dataset we expect to be able to define the cell cluster structure and identify subpopulation markers in the next few months. Once we have these available, we will be able to proceed with all tasks that have been delayed.

3) Potential delay in the *in vivo* part of the pilot treatment study with Selumetinib
The lack of definitive markers for the isolation of specific cell subpopulations will also delay the *in vivo* pilot treatment with Selumetinib, to study the impact on specific cell populations of neurofibromas generated after neurofibromasphere engraftment in nude mice. All technology and expertise are ready to be used for the generation of this *in vivo* neurofibroma model and as soon as markers and Abs for subpopulation analysis are in place, we can start the experiments right away. However, due to this delay, that partially explain also the delay in all ACURO approval, since we centered our efforts in finishing as soon as possible the single cell analysis integration and marker identification, we are foreseeing a potential delay in the finalization of the in vivo experiment that could imply the petition of a non-cost extension of the period of execution of this project. We will better know if we need to ask for a non-cost extension at the beginning of 2024.

6. PROCUCTS

Publications, conferences, papers and presentations

Work presented in conferences during the period of August 2022 – July 2023:

Title: Modeling iPSC-derived neurofibroma-like tumors *in vitro* and *in vivo*
Eduard Serra
Congress: EANO 2022, Vienna (Austria)
17th Meeting & Educational Day Of The European Association Of Neuro-Oncology
Year: 2022 (September 15th-18th)
Place of celebration: In person, Vienna (Austria)
Type of presentation: Invited oral presentation
Single cell data generated in the present project was shown. DOD support was acknowledged (see acknowledgements final slide below).

Title: Modeling Neurofibromatosis Type 1 peripheral nervous system tumors using iPSCs
Eduard Serra
Congress: Meeting der AG Neurofibromatosen, Halle (Germany)
Jährliches Meeting of the AG Neurofibromatosen in Halle an der Saale
Year: 2022 (November 12th)
Place of celebration: In person, Halle (Germany)
Type of presentation: Invited oral presentation
Single cell data generated in the present project was shown. DOD support was acknowledged (see acknowledgements final slide below).

Title: The pNF-ANNUBP-MPNST progression at single cell resolution: a resource for the NF1 community
Bernat Gel/Eduard Serra
Congress: 2023 NF Meeting (Children's Tumor Foundation)
Year: 2023 (24-27 June)
Place of celebration: Scottsdale, Arizona (USA)
Type of presentation: Poster presentation
See the poster attached below.

(see **Section 9. APPENDICES**)

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	Miriam Magallón-Lorenz
Project Role	Pre-doctoral Fellow (bioinformatics)
Researcher Identifier	0000-0003-2741-4572
Nearest person month worked	12
Contribution to the project	Miriam Magallon performed bioinformatic analyses

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**: Singe Cell genomic facility; **Collaboration**: Dr. Holger Heyn Lab
They performed single cell quality control analysis and other QC steps; use of the 10x Chromium platform; 10x library preparation and sequencing; SMART-seq library preparation from sorted cells in a 96-well plate format and sequencing;

Organization Name: Bellvitge Biomedical Research Institute (IDIBELL)
Location of organization: L'Hospitalet de Llobregat (Barcelona), Spain
Partner's contribution to the project: Dr. Conxi Lázaro's group and Dr. Juana Fernandez Rodriguez, director of the IDIBELL Mouse Lab Platform **(Facility).**
Dr. Juana Fernandez Rodriguez is helping with all the paperwork required for the ACURO approval of the in vivo part of this project.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES

25/7/23

Modeling iPSC-derived human neurofibroma-like tumors *in vitro* and *in vivo*

EANO 2022

European Association of Neuro-oncology

Eduard Serra, PhD

Hereditary Cancer Group
Translational Program on **Ca**ncer **Re**search (CARE)
Germans Trias i Pujol Research Institute (IGTP)

Badalona (Barcelona) Spain

1

Acknowledgements

Hereditary Cancer Group-IGTP
(Badalona, Barcelona)

Helena Mazuelas
Míriam Magallón
Itziar Uriarte
Bernat Gel
Meritxell Carrió
Eduard Serra

Cytometry Platform-IGTP
Gerard Requena
Marco-Antonia Fernández

Tumor Bank-IGTP
Christel Kisser Pioch
Laia Pérez-Roca

Hereditary Cancer Group-ICO-IDIBELL
(Hospitalet de LL., Barcelona)

Edgar Creus
Sara Ortega
Juana Fernández-Rodríguez
Alberto Villanueva
Conxi Lázaro

Clinical Genomics Unit Genetics Service-HUGTiP
(Badalona, Barcelona)

Elisabeth Castellanos
Ignacio Blanco

2

Modeling Neurofibromatosis Type 1 peripheral nervous system tumors using iPSCs

Jährliches Meeting der AG Neurofibromatosen in Halle an der Saale am 12.11.2022

25jähriges Jubiläum

Eduard Serra, PhD

Hereditary Cancer Group
Translational Program on **Ca**ncer **Re**search (CARE)
Germans Trias i Pujol Research Institute (IGTP)

Badalona (Barcelona) Spain

1

Acknowledgements

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Clinical Genomics Unit Genetics Service-HUGTiP
(Badalona, Barcelona)

Elisabeth Castellanos
Ignacio Blanco

2

The pNF-ANNUBP-MPNST progression at single-cell resolution: a resource for the NF1 community

Bernat Gel[1], Helena Mazuelas[1], Pere Pericot[1], Miriam Magallón[1], Itziar Uriarte[1], Juan Carlos López-Gutiérrez[2], David Virós[3], Hector Salvador[4], Holger Heyn[5], Meritxell Carrió[1] and Eduard Serra[1]

1) Hereditary Cancer Group, Germans Trias i Pujol Research Institute (IGTP), Badalona, Spain 2) Hospital Universitario Quiron Madrid, Madrid, Spain 3) Hospital Universitary Germans Trias i Pujol (HUGTiP), Badalona, Spain 4) Hospital Sant Joan de Déu, Esplugues de Llobregat, Spain 5) Centre for Genomic Regulation (CNAG-CRG), Barcelona Institute of Science and Technology (BIST), Barcelona, Spain

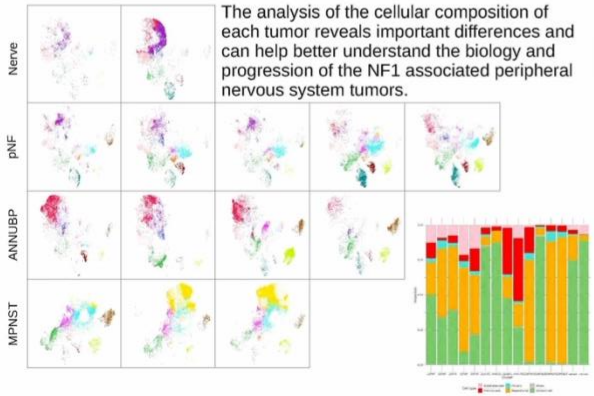
Introduction

Single-cell technologies have revolutionized the study of tumors, providing an unprecedented view into the diversity of cell types and states that can be found inside them. These technologies have evolved rapidly, from the original single-cell RNA-seq to the expansion to epigenomics and more recently spatial transcriptomics, bringing together tissue structure and single-cell gene expression, and they constitute a key component for unraveling tumor complexity.

In the Neurofibromatosis type 1 (NF1) context, there are still few single-cell datasets regarding NF1-related tumors. Particularly, there has been no systematic effort to comprehensively characterize at the single cell level the human NF1-associated peripheral nervous system (PNS) tumors and their path to progression, from a benign plexiform neurofibroma (pNF), to an atypical neurofibromatous neoplasms of uncertain biologic potential (ANNUBP) and finally a malignant peripheral nerve sheath tumor (MPNST).

We present the first multi-modal single-cell catalog of NF1-associated PNS tumors.

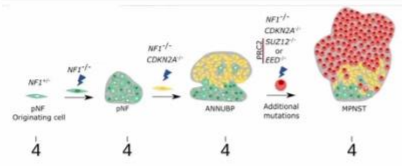
Sample composition



The analysis of the cellular composition of each tumor reveals important differences and can help better understand the biology and progression of the NF1 associated peripheral nervous system tumors.

The resource

Multiple tumor types



Multiple technologies

scRNA-seq - Broad transcriptomic profiles of a large (~10000) number of cells. Only a fraction of each transcript is sequenced. Part of scMultiome.

SMART-seq2 - Detailed and comprehensive transcriptomic profile of a smaller (~100) number of cells. Full transcripts are sequenced.

scATAC-seq - Chromatin accessibility assay for a large (~10000) number of cells. Most informative for small cell clusters. Part of Multiome.

MissionBio (DNA-seq + cell surface markers) - DNA sequencing of a set of genomic regions and quantification of a small number of cell surface proteins.

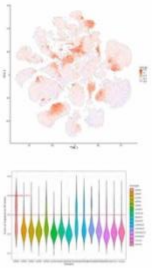
Spatial Transcriptomics - Broad transcriptomic profiles at near-single-cell resolution of tissues.with conserved structure.

Consistent analysis

All samples have been collected and processed following standardized procedures for maximum comparability.

Bioinformatic analysis will be performed using a custom developed data analysis pipeline taking advantage of the latest methods development and benchmarking and will extract the most from each data type.

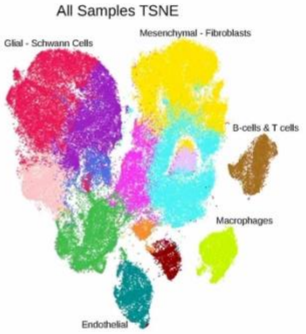
It will include in-house developed methods such as scYoga for the detection and removal of stressed cells which can negatively affect the whole analysis.

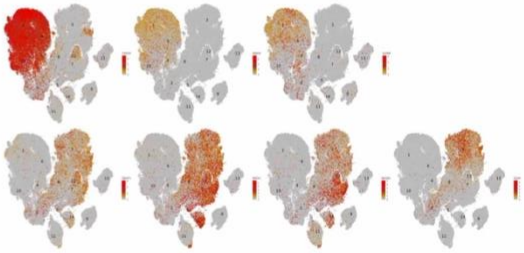


Open resource

All raw and preprocessed data as well as the results of the analysis performed will be freely available to the NF research community trhough the NF Data Portal

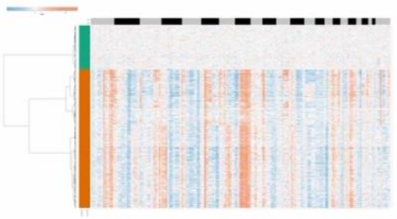
NF DATA PORTAL





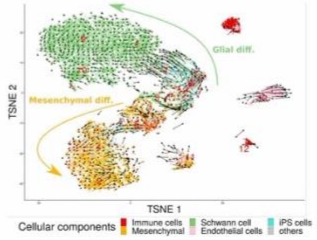
Genomic Heterogeneity

Estimation of genomic copy number alterations can help identify subpopulations in MPNST tumors and study their differences.



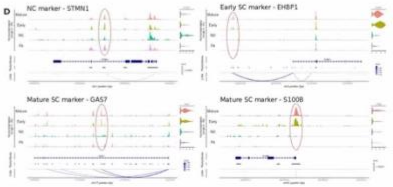
Cellular changes

Advanced analysis such as RNA velocity can help identify cellular changes occurring on the tumors, such as differentiation processes in plexiform neurofibromas.



Integrative analysis

It will be possible to combine the analysis of multiple data types, in multiple individual cells and cell clusters from different tumor types: combining scRNAseq and SMARTseq to identify the clusters with NF1-/- cells, projecting cell clusters on tumor tissue, identifying transcriptional differences between MPNST subpopulations...



This work has been supported by the Congressionally Directed Medical Research Programs (CDMRP) (NF200051)

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