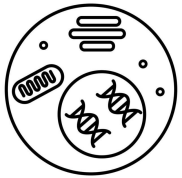


Evaluation and re-analysis of scRNA study on cellular subpopulations from melanoma

Younginn Park

Original study

Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq, Tirosh et al 2016

Exploration of distinct genotypic and phenotypic states of melanoma

Profiling malignant, immune, stromal, and endothelial cells

Melanoma - skin cancer that starts in melanocytes

Massachusetts Institute of Technology

HARVARD MEDICAL SCHOOL

National Institutes of Health (NIH) (.gov)
https://www.ncbi.nlm.nih.gov › articles › PMC4944528

Dissecting the multicellular ecosystem of metastatic ...

by I Tirosh · 2016 · Cited by 3913 — Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. Itay **Tirosh**,[1,*] Benjamin Izar,1,2,3,* ...

Original study design

In total 4645 malignant, immune, and stromal cells isolated from 19 freshly procured human melanoma tumors

10 metastases to lymphoid tissues, 8 to distant sites (e.g. intramuscular tissue and gastrointestinal tract), and one primary acral melanoma.

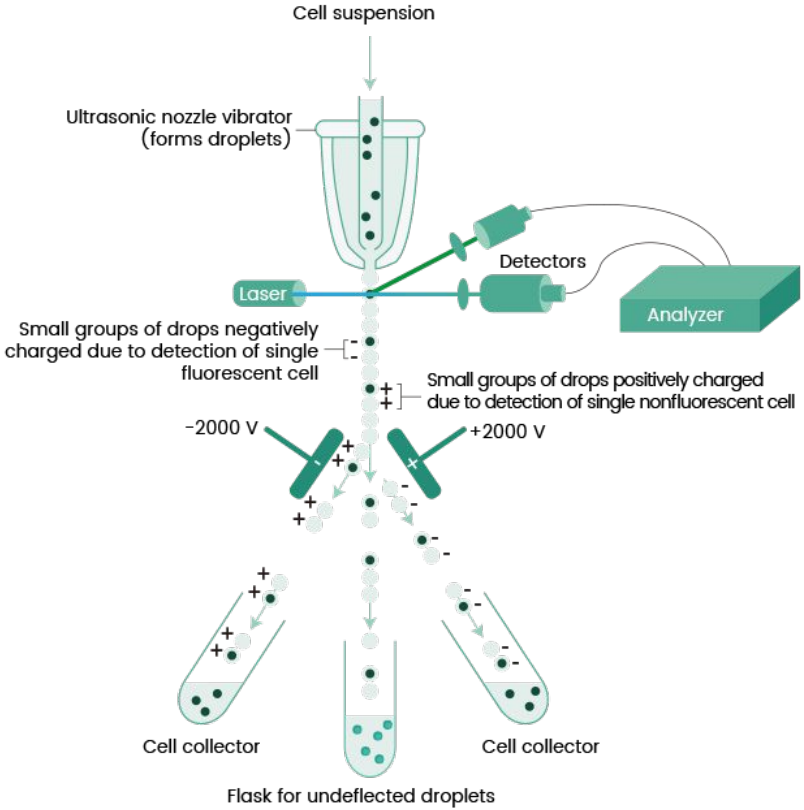
Table S1. Characteristics of patients and samples included in this study

Sample ID	Age/sex	Mutation status	Pre-operative treatment	Site of resection	Post-op. treatment	Alive/ deceased
Melanoma_53	77/F	Wild-type	None	Subcutaneous back lesion	None	Alive
Melanoma_58	67/F	Wild-type	Ipilimumab	Subcutaneous leg lesion	None	Alive
Melanoma_59	80/M	Wild-type	None	Femoral lymph node	Nivolumab.	Deceased
Melanoma_60	69/M	BRAF V600K	Trametinib, ipilimumab	Spleen	None	Alive
Melanoma_65	65/M	BRAF V600E	None	Paraspinal intramuscular	Neovax	Alive
Melanoma_67	58/M	BRAF V600E	None	Axillary lymph node	None	Alive
Melanoma_71	79/M	NRAS Q61L	None	Transverse colon	None	Alive
Melanoma_72	57/F	NRAS Q61R	IL-2, nivolumab, ipilimumab + anti-KIR-Ab	External iliac lymph node	None	Alive
Melanoma_74	63/M	n/a	Nivolumab	Terminal Ileum	None	Alive
Melanoma_75	80/M	Wild-type	Ipilimumab + nivolumab, WDVAX	Subcutaneous leg lesion	Nivolumab	Alive
Melanoma_78	73/M	NRAS Q61L	WDVAX, ipilimumab + nivolumab	Small bowel	None	Deceased
Melanoma_79	74/M	Wild-type	None	Axillary lymph node	None	Alive
Melanoma_80	69/F	NRAS Q61L	None	Axillary lymph node	None	Alive

Single cell isolation method

Individual cells were isolated using FACS (Fluorescence-activated Cell Sorting, type of flow cytometry), this process included manual removal of doublets

SMART-Seq2 was used for amplification and Illumina NextSeq 500 for sequencing



https://www.sinobiological.com/category/fcm-facs-facs

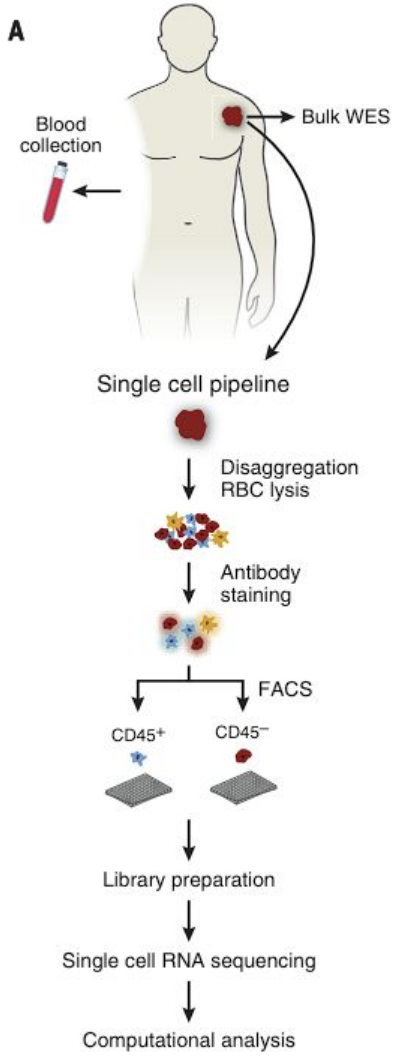


Diagram of the basic workflow

scRNA-seq data

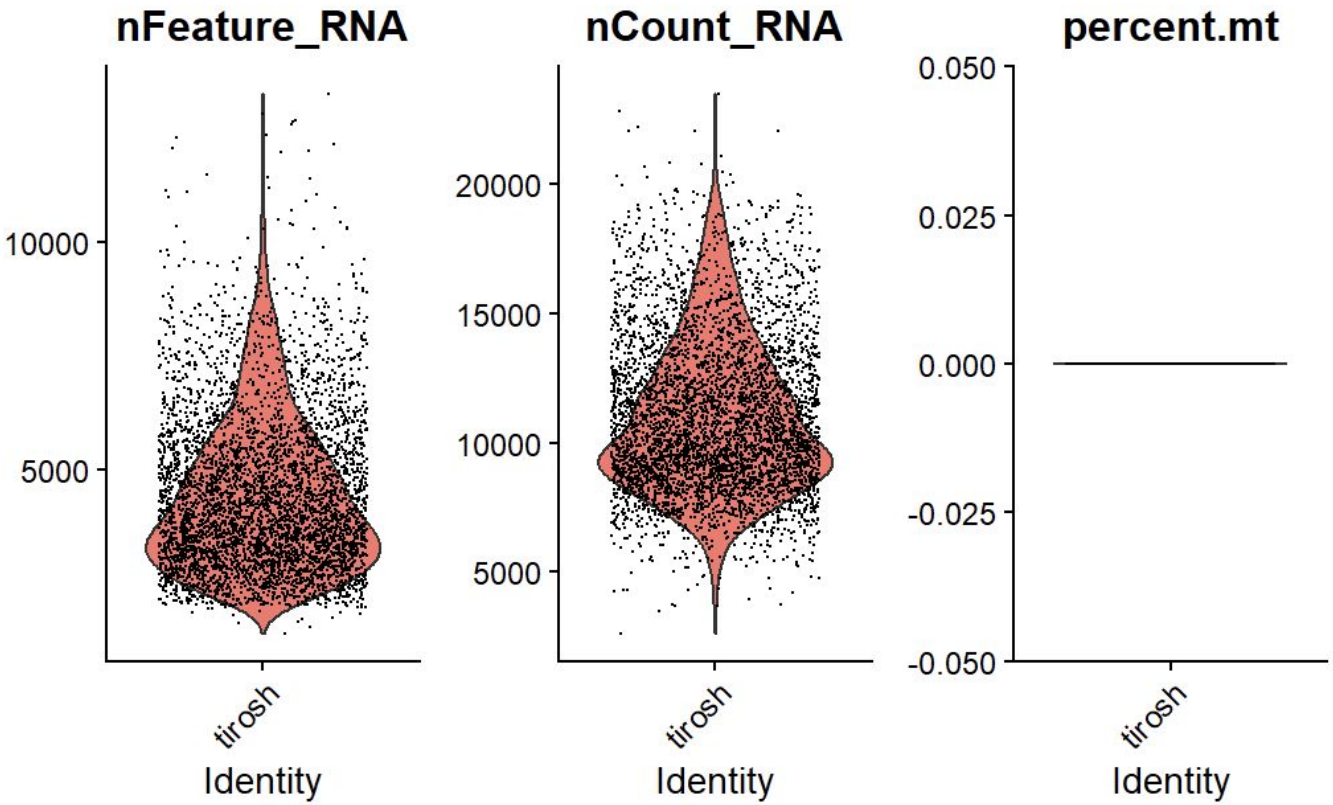
The scRNA-seq data provided by the authors (GSE72056) was already processed in many ways - basic normalization (TPM), cells were filtered by the number of detected genes (min. 1,700)

Expression matrix = 4,645 cells x 23,686 features

3,256 non-malignant cells, 1,257 malignant cells

No information about technical variables

Metadata - tumor ID, if malignant, type of immune cell


nFeature_RNA
10000
5000
tirosh
Identity
nCount_RNA
20000
15000
10000
5000
tirosh
Identity
percent.mt
0.050
0.025
0.000
-0.025
-0.050
tirosh
Identity

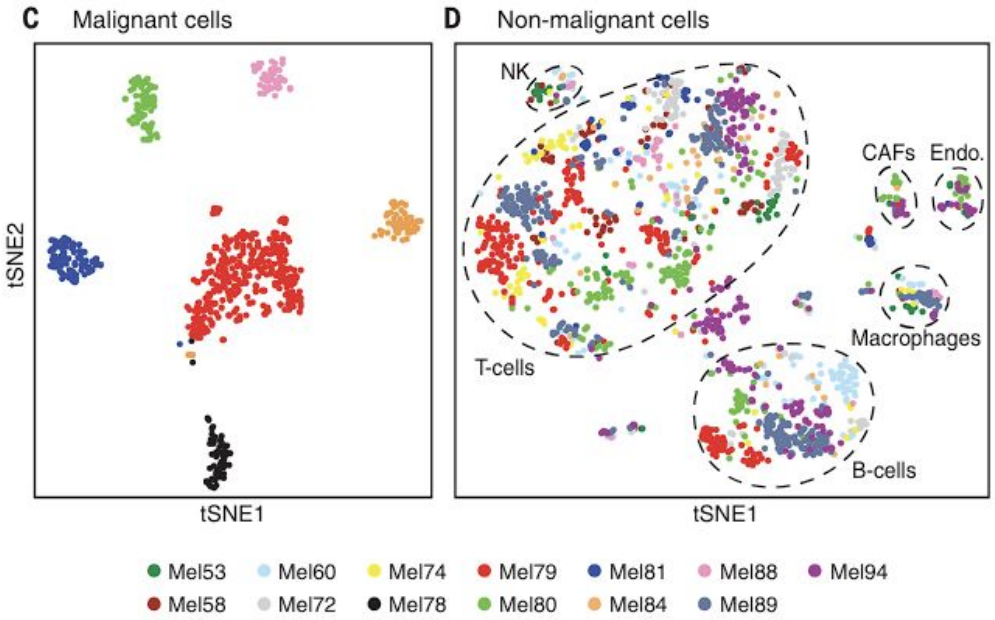
Methods - cluster analysis

t-SNE (dim=15, Matlab implementation), marker identification and visualization, clustering using DBscan

“Since the complexity of tSNE visualization increases with the number of tumors we **restricted the analysis to the 13 tumors with at least 100 cells**, and for the malignant cell analysis we further **restricted the analysis to 6 tumors with >50 malignant cells**.”

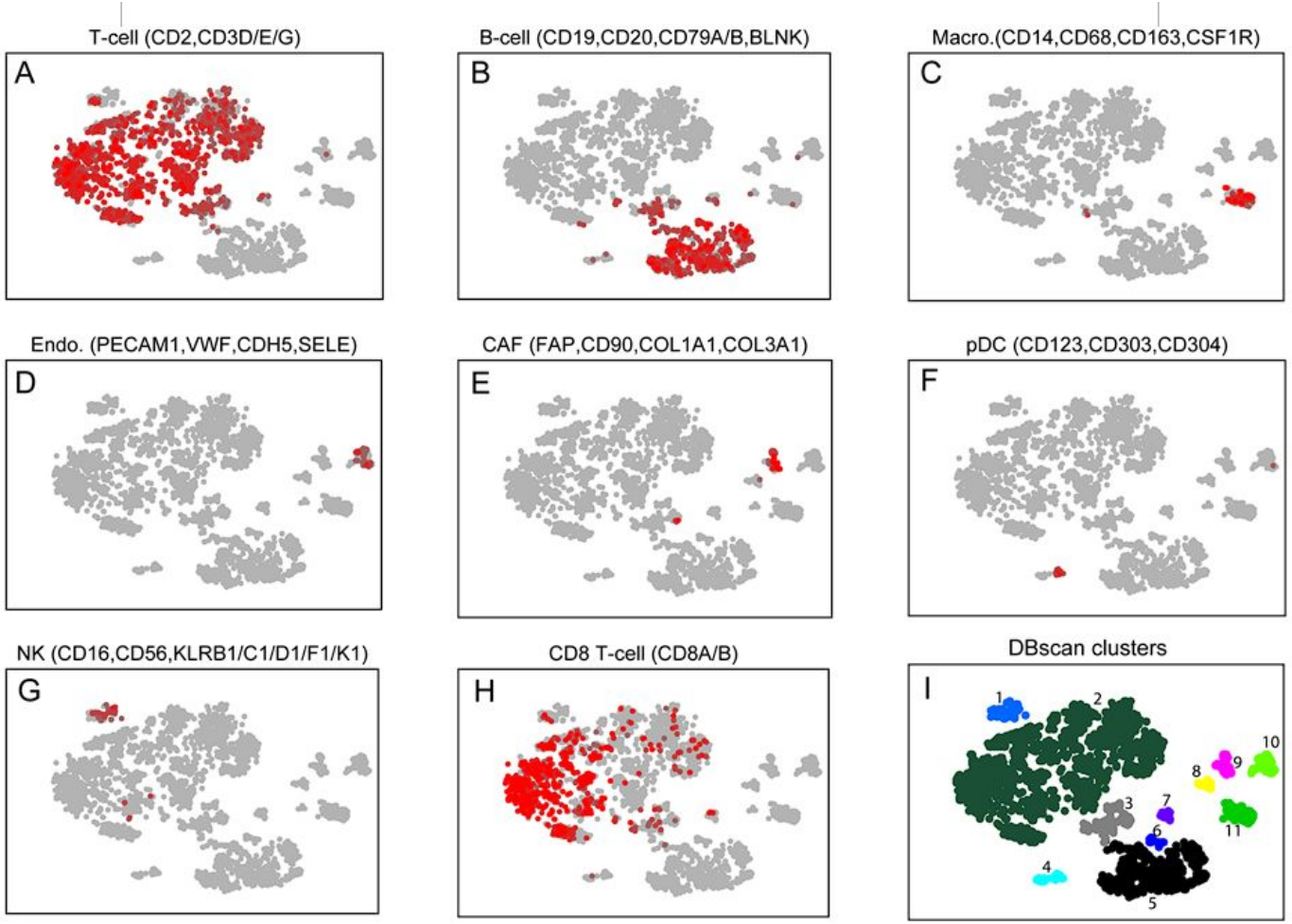
Results - cluster analysis

"To define cell types from the non-malignant tSNE analysis we used a **density clustering method, DBscan**. This process revealed **six clusters** for which the top preferentially expressed genes included multiple known markers of particular cell types."

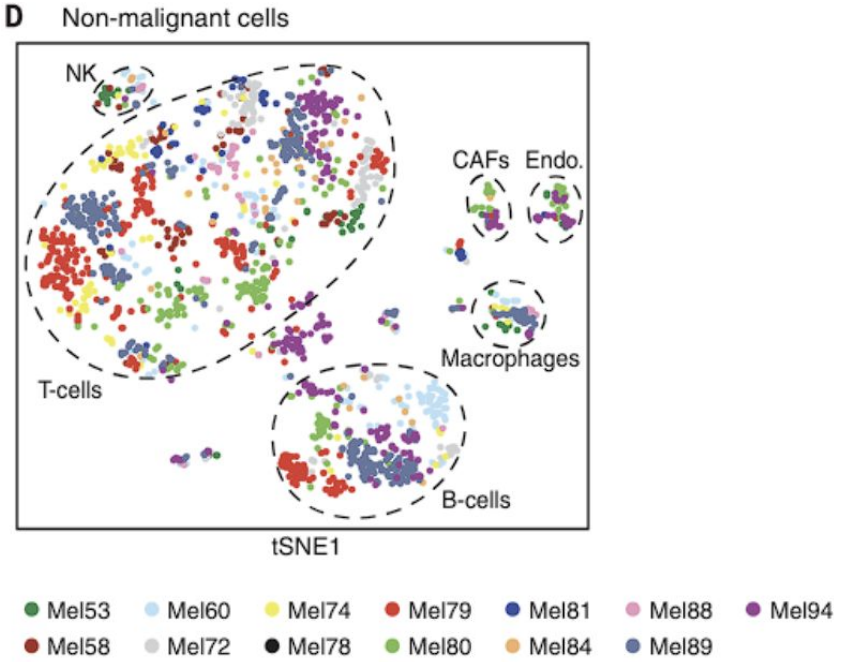


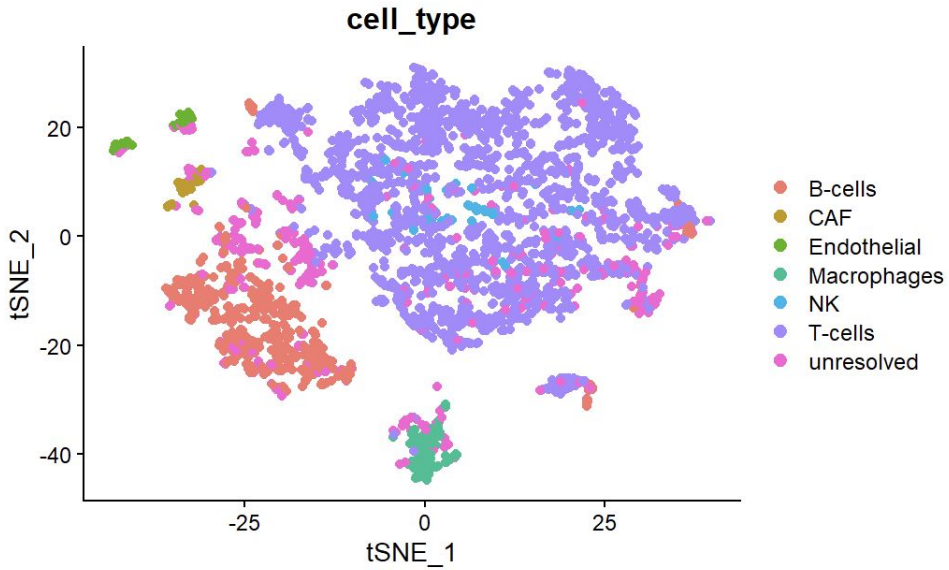
Results of t-SNE run on malignant cells (left) and non-malignant cells (right

Results

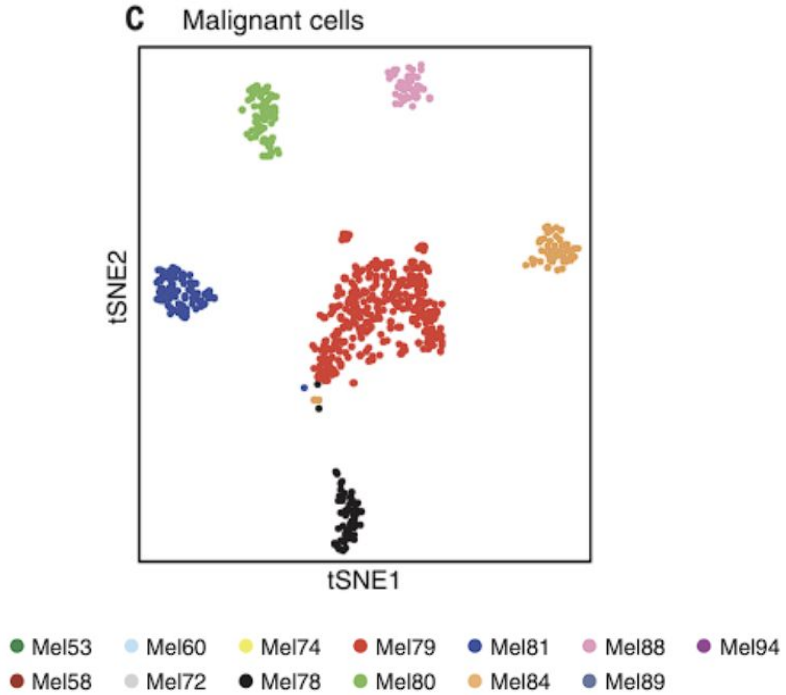

T-cell (CD2,CD3D/E/G)
A
B-cell (CD19,CD20,CD79A/B,BLNK)
B
Macro.(CD14,CD68,CD163,CSF1R)
C
Endo. (PECAM1,VWF,CDH5,SELE)
D
CAF (FAP,CD90,COL1A1,COL3A1)
E
pDC (CD123,CD303,CD304)
F
NK (CD16,CD56,KLRB1/C1/D1/F1/K1)
G
CD8 T-cell (CD8A/B)
H
DBscan clusters
I
1
2
3
4
5
6
7
8
9
10
11

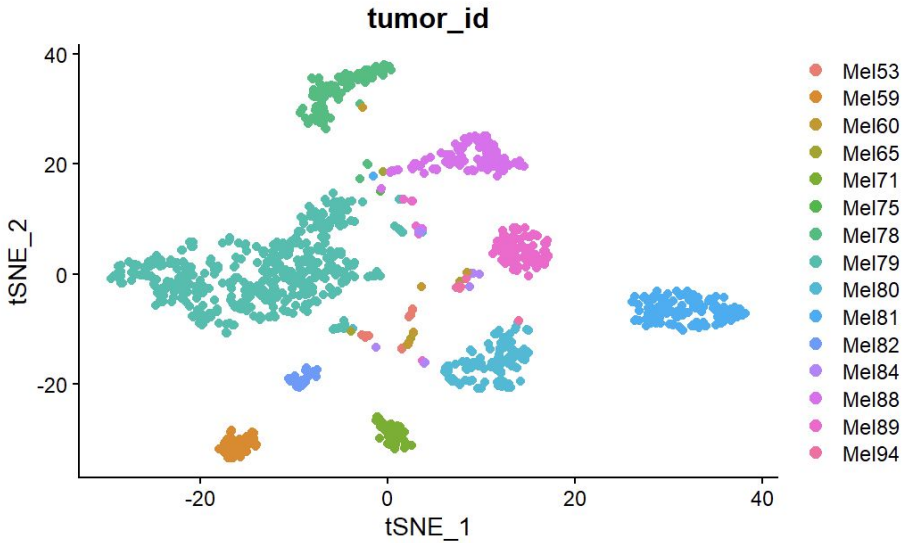
Reconstruction - non-malignant cells





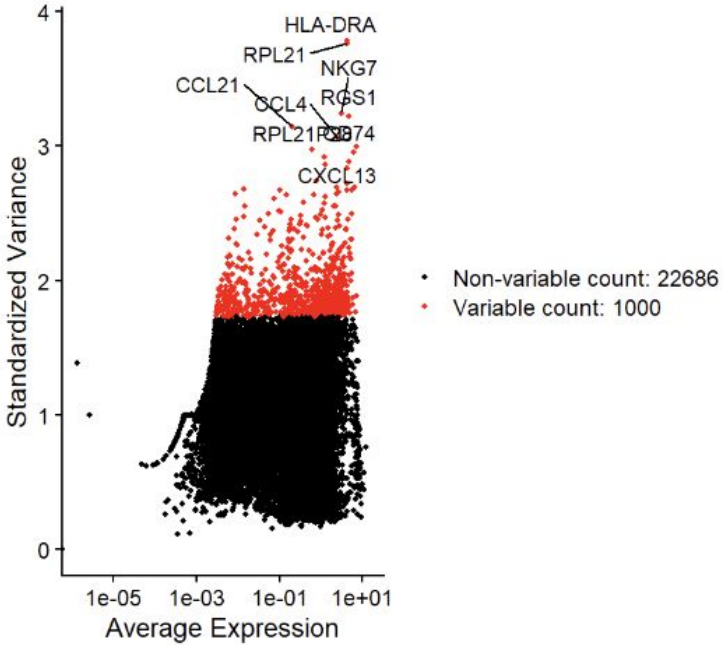
Reconstruction - malignant cells



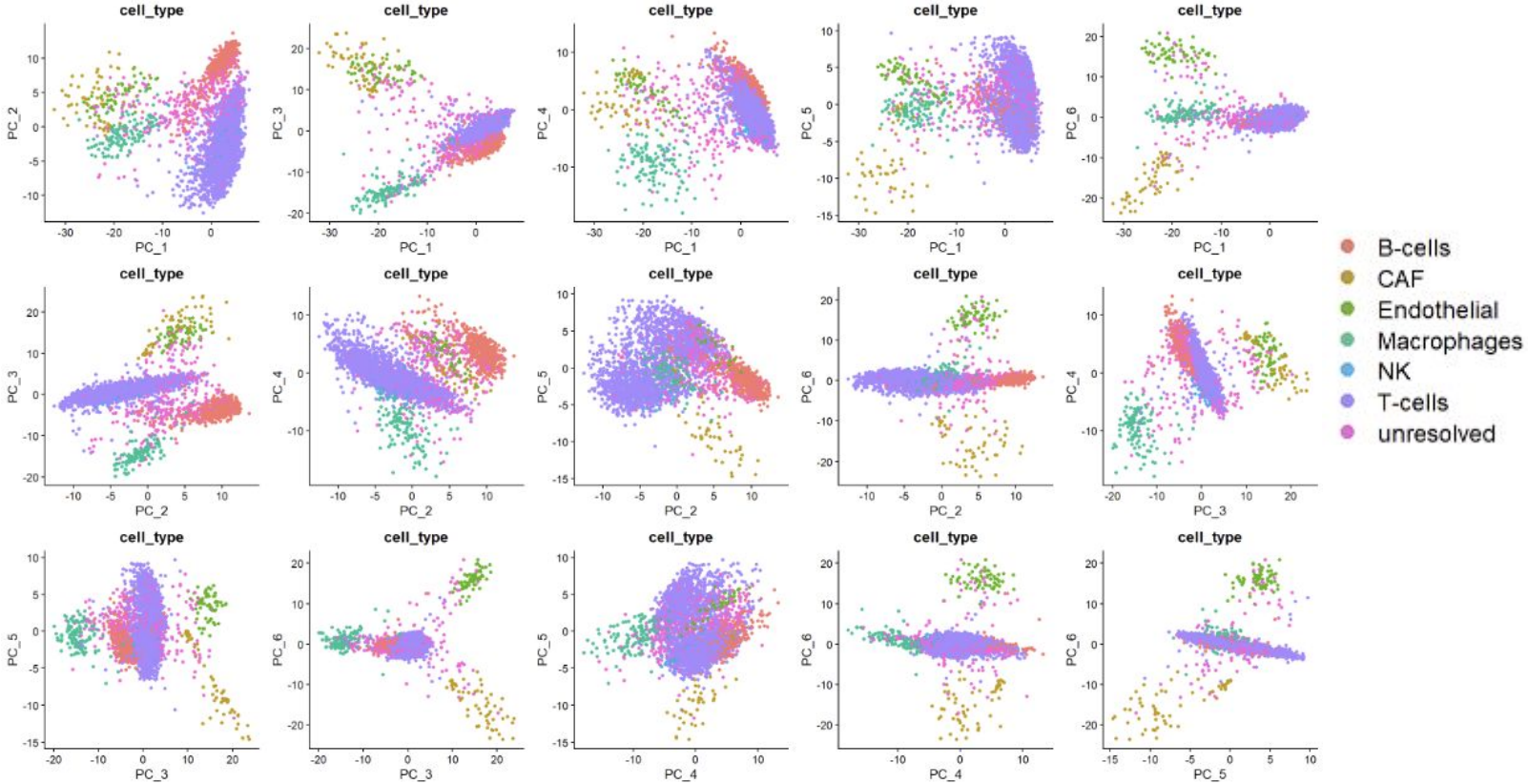


Improvement - basic feature selection

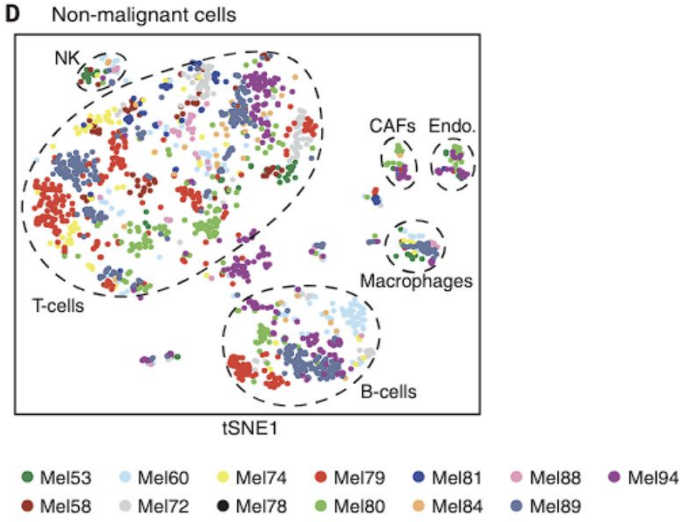
Most variable 1,000 features (vst method) analysis in Seurat

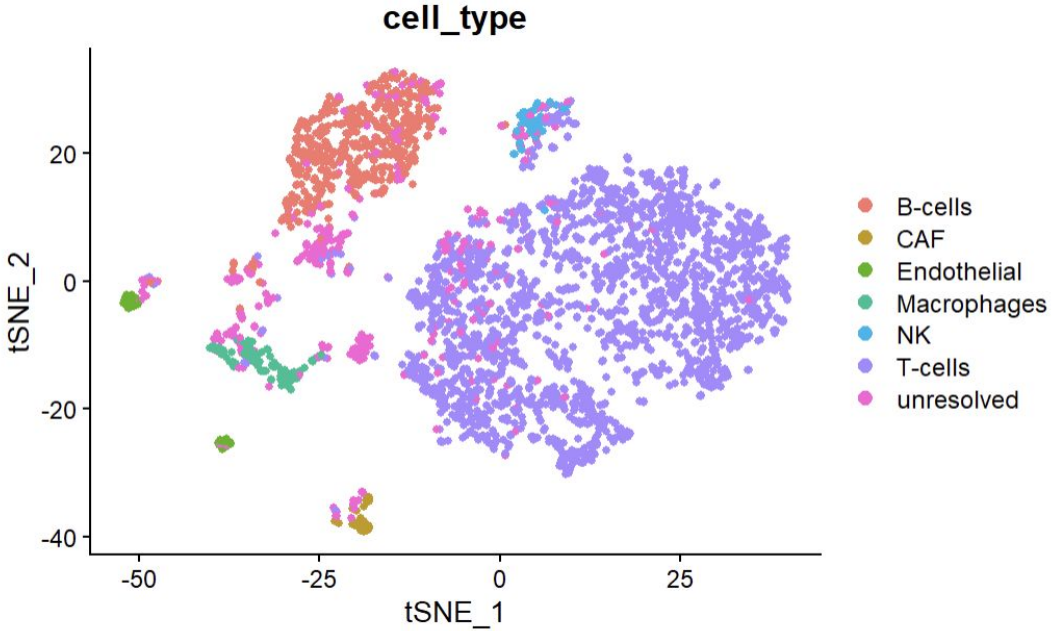


PCA after VariableFeatures



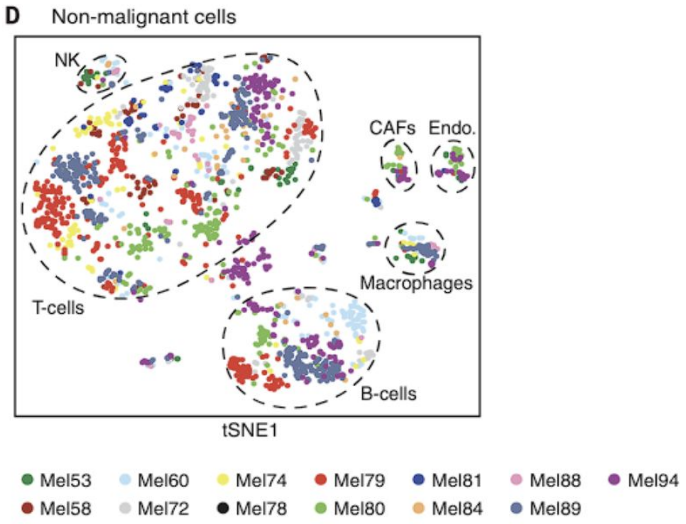
t-SNE after VariableFeatures

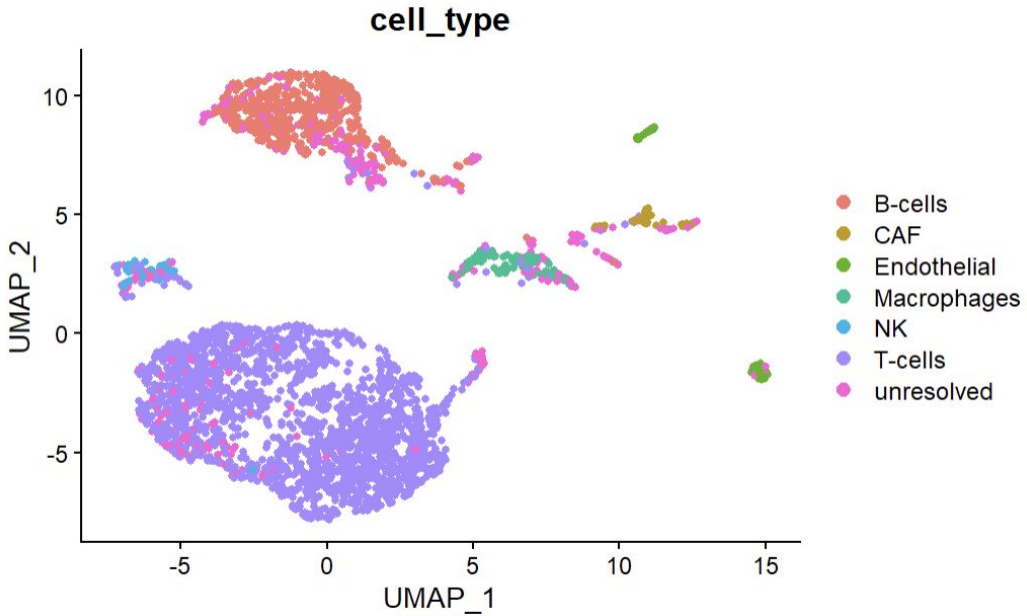




UMAP after VariableFeatures

Different n_neighbors were tested (=10 gave visually the best results)





Discussion - cluster analysis

Improvement from the original study - all tumor sample data was preserved, complete view of inter-tumor differences

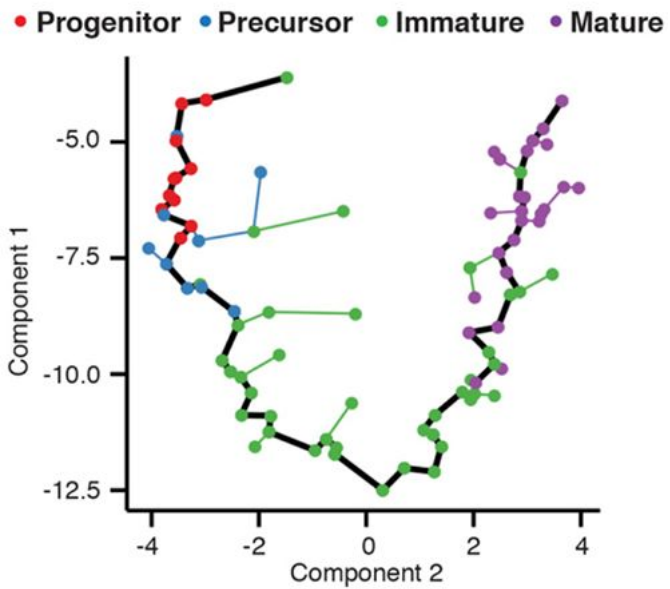
Jackstraw feature selection might be worth considering for better feature selection

Trajectory analysis

Cells in a sample can display differing states even if they come from the same population undergoing the same process.

Time-related (temporal) processes have inherently described cell “trajectories” in them

Problem definition: order the cells in a sample in pseudo-time (units of stages in a process) using clustering and graph methods



Trajectory of olfactory neuron development in mice, https://cole-trapnell-lab.github.io/projects/sc-trajectories/

Original study

“[...] all tumors harbored malignant cells from **two distinct transcriptional cell states**, such that tumors characterized by high levels of the **MITF transcription factor** also contained cells with low MITF and elevated levels of the **AXL kinase**”

Immunohistochemistry analysis

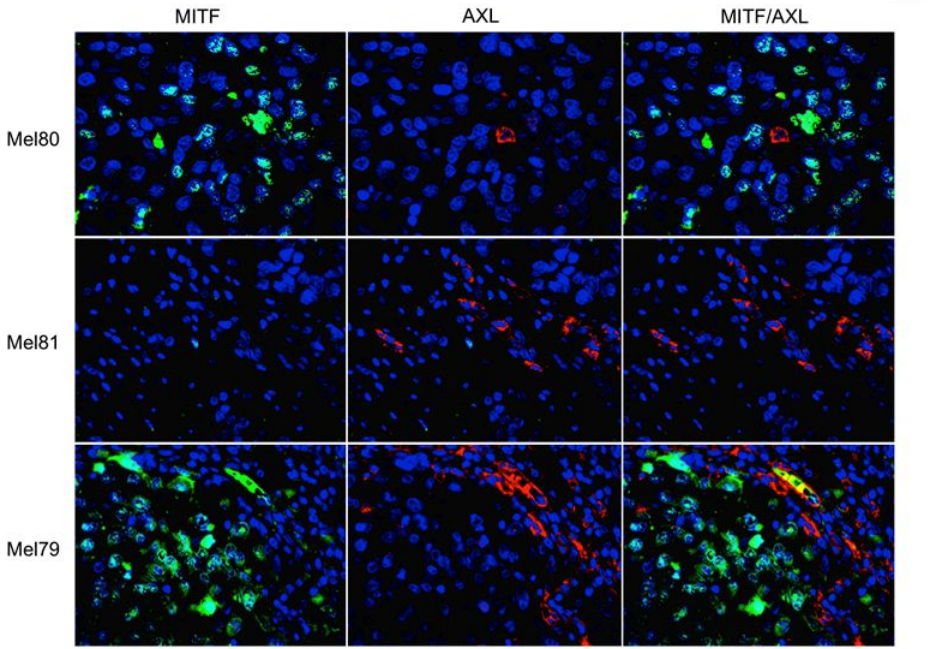


Figure S8. AXL/MITF immunofluorescence staining of tissue slides of Mel80, Mel81 and Mel79 (40x magnification) revealed presence of AXL-expressing and MITF-expressing cells in each sample. Consistent with single-cell RNA-seq inferred frequencies of each population, Mel80 contained rare AXL-expressing cells (red, cell membrane staining) and mostly malignant MITF-positive cells (green, nuclear staining), while malignant cells of Mel81 almost exclusively consisted of AXL-expressing cells. Mel79 had a mixed population with rare cells positive for both markers, all in agreement with the inferred single-cell transcriptome data.

Biological context - melanoma profiles

Two transcription profiles that were being focused on for malignant cells - **MITF** and **AXL** - both have great influence on prognosis, treatment response; both oncogenes

MITF - regulator in melanoma, crucial for the development, differentiation, and survival of melanocytes

AXL - invasive and metastatic behavior of melanoma, attractive target for cancer therapies

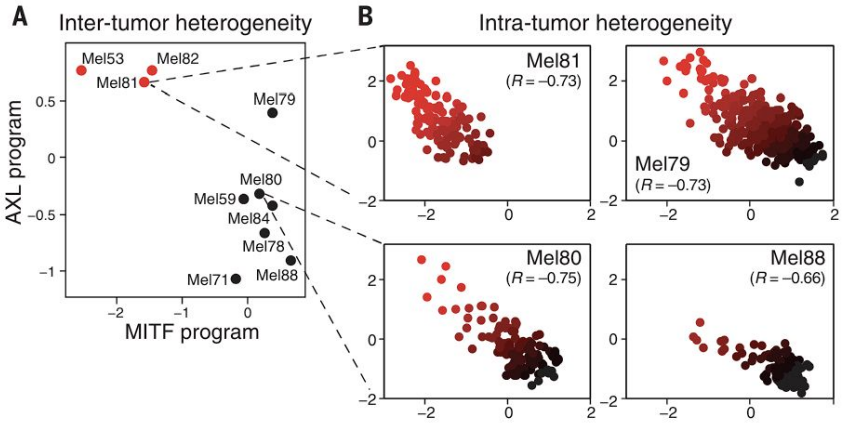
Inverse correlation - High MITF levels are typically associated with low AXL expression (high proliferation, low metastasis cancer phenotype) and vice versa

PCA and correlation study

“PC2-6, which were associated with the cell cycle (PC2 and 6), regional heterogeneity (PC3) and MITF expression program (PC4 and 5).”

“The **top 100 MITF-correlated genes** across the entire set of malignant cells were defined as the **MITF program**, and their average relative expression as the **MITF-program cell score**. The average expression of the **top 100 genes that negatively correlate with the MITF program scores** were defined as the **AXL program** and used to define AXL program cell score.”

Inter- and intra-tumor heterogeneity



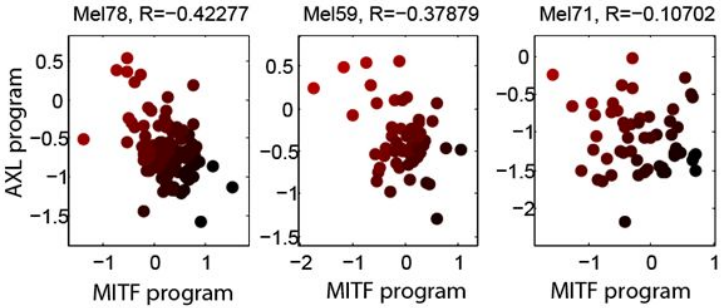
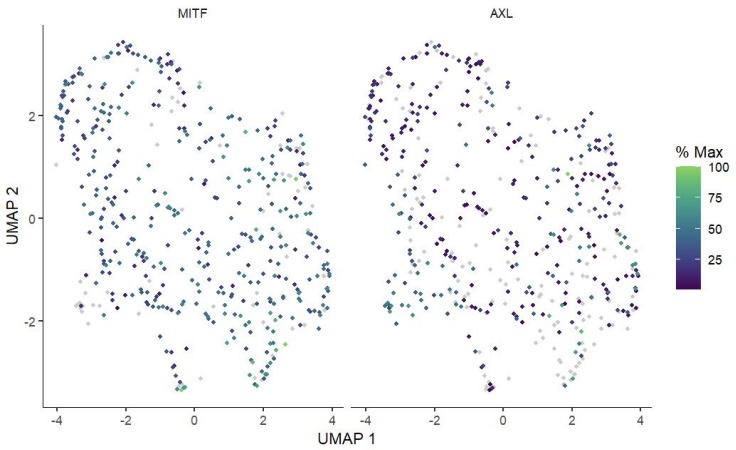


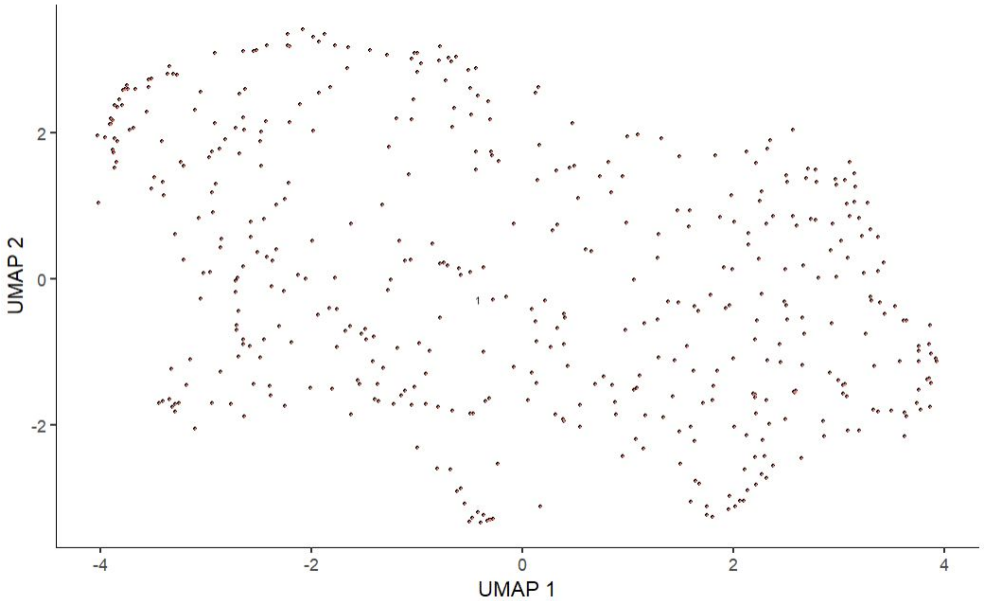
Figure S7. Intra-tumor heterogeneity in AXL and MITF programs. AXL-program (Y-axis) and MITF-program (X-axis) scores for malignant cells in each of the three tumors with a sufficient number of malignant cells (n>50) that were not included in Fig. 3B. Cells are colored from black to red by the relative AXL and MITF scores. The Pearson correlation coefficient is denoted on top.

Analysis on Mel79

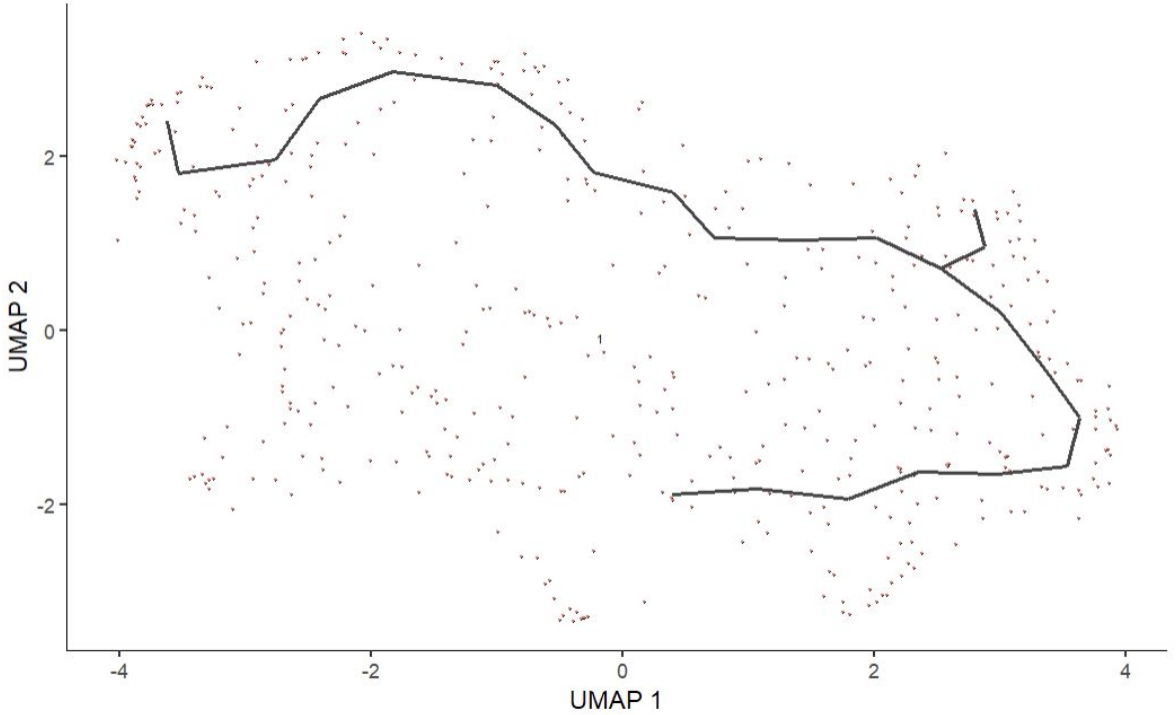
UMAP in Monocle3

First challenge - no clear intra-cellular clusters/heterogeneity

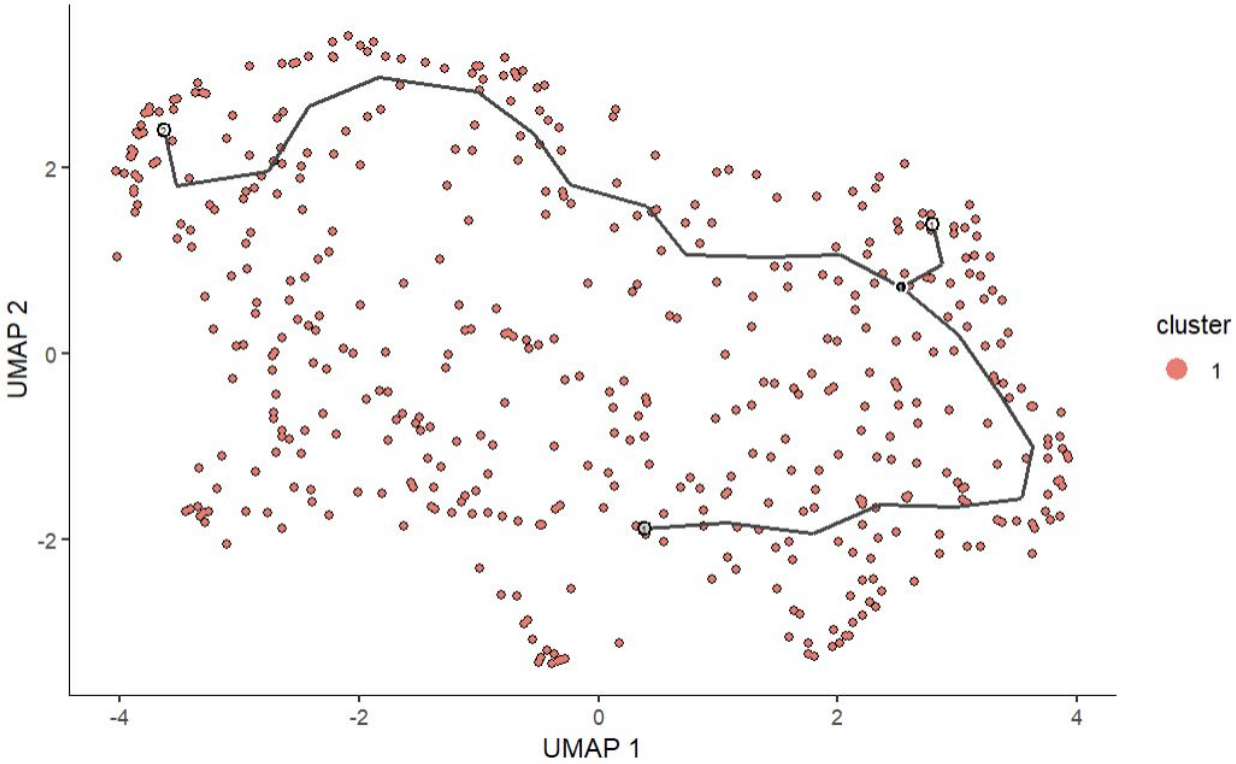




Learning the graph



Learning the trajectory



Discussion - trajectory analysis

The user has to input the beginning node in the learned path manually - for now I was mostly going in to this analysis blindly

Might be worth considering different dimension reduction methods

Consider feature selection - like the one used in the previous section

Possible further enhancement

The data/cell count wasn't too great for a single tumor sample (Mel79 - 468 malignant cells, which is roughly a third of all malignant cells in the dataset)

The original study also analyzed cytotoxic and exhaustion programs in T-cells (Mel75) - can also be treated as a trajectory problem in an analogous way, although the are more cell-to-cell interaction aspect that have to be taken into consideration

References

Tirosh I, Izar B, Prakadan SM, Wadsworth MH 2nd, Treacy D, Trombetta JJ, Rotem A, Rodman C, Lian C, Murphy G, Fallahi-Sichani M, Dutton-Regester K, Lin JR, Cohen O, Shah P, Lu D, Genshaft AS, Hughes TK, Ziegler CG, Kazer SW, Gaillard A, Kolb KE, Villani AC, Johannessen CM, Andreev AY, Van Allen EM, Bertagnolli M, Sorger PK, Sullivan RJ, Flaherty KT, Frederick DT, Jané-Valbuena J, Yoon CH, Rozenblatt-Rosen O, Shalek AK, Regev A, Garraway LA. Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. Science. 2016 Apr 8;352(6282):189-96. doi: 10.1126/science.aad0501. PMID: 27124452; PMCID: PMC4944528.