JSEQ® manual

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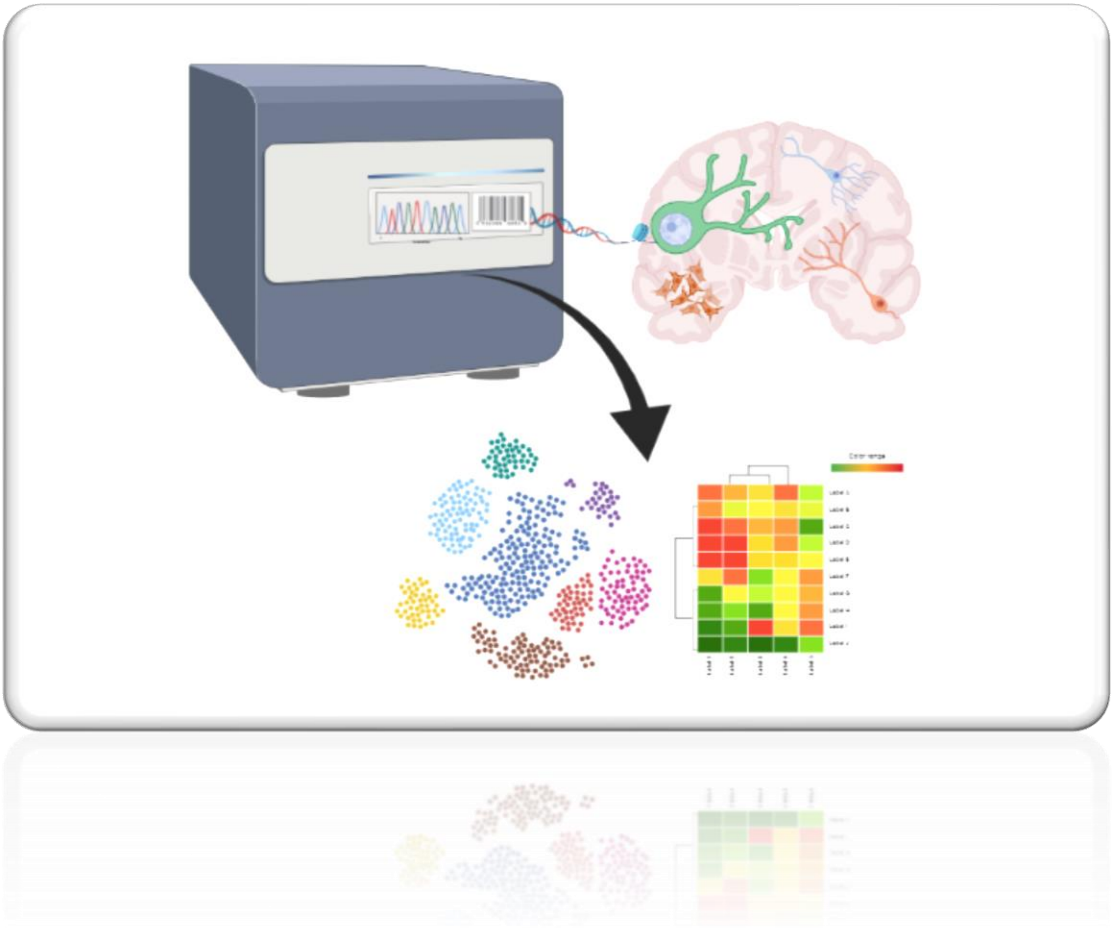
JSEQ® - single cell sequencing analysis tools



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Introduction

Single-cell sequencing is a modern technique of sequencing at the single-cell level. Such an approach gives a lot of possibilities to get knowledge about gene expression in different cells populations during development, carcinogenesis or after treatment with novel gen therapy. But how it works? Actually, so-called single-cell sequencing does not focus on 'single cell' but on whole populations of cells which we sequence. So whence obtain we information about single cell? Currently, we have some approaches for single-cell sequencing but the final effect is similar. Results of single-cell sequencing is a great number of cell transcriptome sequences with special sequence signs as 'barcode' and 'UMI' - Unique Molecular Identifiers. The barcode sequence is different for each cell during sequencing. Furthermore 'UMI' is independent of the barcodes sequence which allows distinguishing each copy of mRNA (count) in the cell (for each barcode) and removing duplicated values. So there are two important sequences in bioinformatics single-cell analysis, barcode (about 12 bp) specific to the single cell, and UMI (about 8 bp) specific to the single copy of mRNA (count) in every single cell. Due to this, we can talk about 'single cell' sequencing although during sequencing and analysis we have thousands of cells' transcriptomes. After sequencing, obtained results are conducted by multi-stage quality control, statistical analysis (eg. clustering algorithm PCA, kNN, sNN and visualization support algorithm by dimensionality reduction tSNE and UMAP). The JSEQ® fully automatic pipeline provides an easy and fast way to obtain high-resolution results for single cells using various types of input.

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1. First use and installation

The JSEQ® pipeline was prepared for UNIX/LINUX systems, so if you WINDOWS user and you have Windows 10, don't worry. Currently, Microsoft® offer a good LINUX subsystem without change the basic WINDOWS system. So if you still haven't the LINUX subsystem on your PC, go to Microsoft Store and install Ubuntu LTS and enjoy your first steps in the UNIX environment. If your UNIX environment is ready to go to my GitHub.

1.1. Working directory

In the JSEQ® 2.1.0 release were added option to choose where the pipeline required tools should be installed. If you choose 'local' then the whole pipeline will be installed at localhost or when you choose 'docker' the requirements will be installed in the docker container (before using this option install docker on your computer). In both cases project, results and requirement files you can view and modify on localhost and only analysis and tools installation will be conducted in the container in case of the 'docker' working directory.

1.1.1. Open a right directory in bash console and clone git repository:

git clone https://github.com/Qubix96/JSEQ_scRNAseq.git

1.1.2. Go to the JSEQ_scRNAseq repository and run programme by writing './JSEQ' in the console

1.1.3. Choose a working directory: localhost write 'local'; docker write 'docker' in the console

```
Welcome in the JSEQ® scRNAseq pipeline which was prepared at Institute of Bioorganic Chemistry, Polish Academy of Sciences in Poznań
All information and references you can check in the file JSEQ manual on my GitHub
Contact: jkubis@ibch.poznan.pl or jakub.kubis1996@gmail.com


Choose JSEQ working directory location:
Local [local]
Docker container [docker]
```

1.2. Installation

1.2.1. Write the command 'install' in the console and push enter

Docker container:

```
Choose function:
-docker container installation [install]
-start container [start]
```

Localhost:

```
Choose JSEQ function:
-local installation [install]
-download RefGenome (GENCODE) [genome]
-creat new project [project]
-start analysis [analysis]

Choose function:
```

***There you can see all options what give JSEQ pipeline.**

1.2.2. In case of localhost write ‘sudo’ to give permission for installing all the required files

```
Write [sudo] to give installation permission
sudo
```

1.2.3. Wait for installation finish. You will see the installation progress in the console but if you want to check that there weren’t any errors, go to JSEQ_scRNAseq/setup/install.log.out and explore results

1.3. First use

If the JSEQ were installed you can get to know about the rest options other than ‘install’. There were described shortly each of them in the next chapters, where you can find how they work and how to use them.

I would like to point out that JSEQ can be used for analysis at different steps. The first option gives possibilities to start the analysis from FastQ files. When you want to run it, firstly you should choose the option ‘download RefGenome’. In this option, a reference genome and gene annotations will download from https://www.gencodegenes.org/ website. In this version of JSEQ, you can choose the Human or Mouse genome. If you will need other genomes, you can write an email to me (email in contact).

Another option is to run an analysis when we have results after mapping raw reads to the genome. It can be data in the format of the matrix (or sparse matrix) for raw counts or normalized expression values. However, before running the analysis, you have to create a project. To do this you have to run the option ‘ create new project’, there are two options to choose and they will depend on which analysis you choose: raw data (fastq files) or after mapping. The same will regard to ‘start analysis’ option where you will have to choose analysis options.

After analysis in both cases, you will obtain reports: one report with single-cell results in option without mapping and two reports for raw data analysis (report for quality control of sequences and report with single-cell analysis results).

1.4. Working in docker:

1.4.1. Run docker container: write ‘start’ in console

```
Choose function:
-docker container installation [install]
-start container [start]
```

Since this moment all the rest functions will be the same as were written below for the local working directory. What is more, results are saved in the local repositories. Requirements file like markers and information about UMIs and barcodes length you can also modify in the local repository.

```
Docker container


Choose JSEQ function:
-download RefGenome (GENCODE) [genome]
-creat new project [project]
-start analysis [analysis]

Choose function:
```

2. Analysis – raw data (fastq files)

2.1. Genome download

Before start analysis, you have to download reference genome and gene annotations proper to your data sets. The downloaded genome will be deposited in a separate directory from your analysis directory. What is more, you will have to annotate genes to your genome but this step will run during project creation because gene annotation depends on your sequenced reads length (eg. for Nextera550 is 75 or150 bp). This step can last a while, so to prevent lost time each time when you create a new project, the annotated genome is saved also in a different directory than your project directory. When you will run an analysis in future with the same length of the reads, the gene annotations step will be omitted.

Genome downloading:

2.1.1. Write 'genome' in the console and push enter

2.1.2. Choose specie for which will you run the analysis. Write in console right name. There is also an option for mixed mouse/human analysis prepared for mixed data experiments. When you write 'mix' in the console will download both genomes

2.2. Create project

Now you have to create a new project for raw reads fastq analysis. How I wrote above during this step, will be run gene annotation which depends on your reads length.

Project creating:

2.2.1. Write ‘project’ in the console and push enter

```
Choose JSEQ function:
-first installation [install]
-download RefGenome (GENCODE) [genome]
-creat new project [project]
-start analysis [analysis]

Choose function:

project
```

2.2.2. To choose ‘fastq data’ write ‘1’ in the console

```
Choose function:

project
Create project for fastq data [1] or counts / expression data  [2]:
1
```

2.2.3. Now you have to fill in some data as project name, reads length, species, the estimated number of cells

Project name – write your project name, when your name includes more than one words then use ‘_’ instead of ‘space’.

Reads length – write reads length depends on your sequencing technology.

Estimated number of cells – write the number of cells how you expect with your single-cell technology (eg. dropSEQ).

Marker set – choose right markers set (default set of markers is for the brain cell types) which will be used in current for this repository analysis.

```
Project name:

test1
Enter operation paramets:

ReadsLenght:

75
Species [human/mice/mix]:

mice
Estimated number of cells

5000
Choose marker set

1) /mnt/c/GIT_Projekty/JSEQ_scRNAseq/requirements_file/markers.xlsx
#?
```

When all parameters will correct, you will see the message and source where you should put your FastQ data. It can be multiple data, which will be combined (eg. 5x Read1.fq and 5x Read2.fq).

```
Complete

Next step =>


Put fastQ data into => projects/23-02-2021_manual_fq/fast_data
If you done it, push ENTER
```

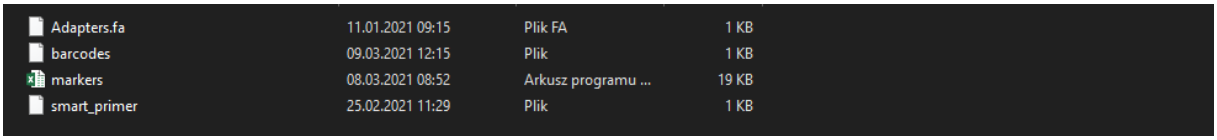
2.2.4. Put data into the given source directory

Remember that all Read1 files should contain in filename '_R1' and Read2 '_R2'. Without that, you will not go to the next step!

2.3. Run analysis

When your project and data is ready you can run the first analysis.

Make sure that used markers, adapters, smart primer sequence and barcode and UMI length is proper for your analysis. Go to JSEQ_scRNAseq/requirements_file/* and check this.



Adapters:

Open adapters file and check adapters sequence.

```
>CUSTOM_P5_INDEX_SUFIX
GCCTGTCCGCGG
>P5_INDEX_SEQUENCE
AATGATACGGCGACCACCGAGATCTACACGCCTGTCCGCGG
>P5_INDEX_SEQUENCE_PREFIX
AATGATACGGCGACCACCGAGATCTACAC
>ILLUMINA_UNIVERSAL_READ_SEQUENCE
AGATCGGAAGAG
>CUSTOM_READ_SEQ_REVERSE_COMPLEMENT
GTACTCTGCGTTGATACCACTGCTTCCGCGGACAGGC
>INDEX_NXX7_PREFIX
GTCTCGTGGGCTCGG
```

*if your you need more or other adapters, so change or add them

Barcodes:

Open barcodes file and check length for UMI and barcodes.

```
barcode_start=1
barcode_end=12
umi_start=13
umi_end=20

barcode='(?P<cell_1>.{'$barcode_end-$barcode_start+1'})(?P<umi_1>.{'$umi_end-$umi_start+1'})'
```

Barcode length

UMI length

*if your single-cell technique has other lengths, so change them

Smart primer:

Go to smart_primer file and check primer sequence

```
1  smart=AAGCAGTGGTATCAACGCAGAGTAC
```

*if your single-cell technique has other smart primer, so change it

Markers:

Go to file markers.xlsx and check markers.
How works cell naming with markers, you can read in part **5.10 Cells naming – markers**

Fastq analysis include:

- Trimming adapters and quality controle of reads
- Reads mapping proces
- Createing required files in analysis (whitelists, genome dictionary file)
- UMI and barcodes extraction
- Barcodes repairing
- Creating gene count matrix
- Statistical analysis
- Reports generate

Analysis:

2.3.1. Write 'analysis' in the console and push enter

```
Choose JSEQ function:
-first installation [install]
-download RefGenome (GENCODE) [genome]
-creat new project [project]
-start analysis [analysis]

Choose function:

analysis
```

2.3.2. To choose 'fastq data' write '1' in the console

```
analysis
Analysis of data for fastq analysis project [1]  counts / expression analysis project [2]:
```

2.3.3. Choose a project which you want to analyse. Write the project number and push enter

```
analysis
Analysis of data for fastq analysis project [1]  counts / expression analysis project [2]:
2
1) /mnt/c/GIT_Projekty/JSEQ_scRNAseq/projects/28-06-2021_test1_exp
2) /mnt/c/GIT_Projekty/JSEQ_scRNAseq/projects/28-06-2021_test2_exp
3) /mnt/c/GIT_Projekty/JSEQ_scRNAseq/projects/28-06-2021_test3_exp
#?
```

2.3.4. It can last a while, some information you can see in the console, but if you want to check that all works correctly, go to projects directory 'project/*project_name*/results/process.log.out'. You can checking and refreshing this log file in real-time during analysis. In the same directory, you can see reads quality control report and statistical single-cell analysis report

3. Analysis – gene count / normalized expression

During this analysis you needn't download any genome and run gene annotation, because your data should be in form of gene count / normalized expression matrix or sparse matrix.

3.1. Create project

Project creating:

3.1.1. Write 'project' in the console and push enter

```
Choose JSEQ function:
-first installation [install]
-download RefGenome (GENCODE) [genome]
-creat new project [project]
-start analysis [analysis]

Choose function:

project
```

3.1.2. To choose 'counts / expression data' write '2' in the console

```
analysis
Analysis of data for fastq analysis project [1]  counts / expression analysis project [2]:
```

3.1.3. Now you have to fill in some data as project name, species, the estimated number of cells

Project name – write your project name, when your name includes more than one words then use '_' instead of 'space'.

Estimated number of cells – write the number of cells how you expect in your data.

Marker set – choose right markers set (default set of markers is for the brain cell types) which will be used in current for this repository analysis.

```
Project name:

test1
Enter operation paramets:

Species [human/mice]:

human
Estimated number of cells

1000
Choose marker set

1) /mnt/c/GIT_Projekty/JSEQ_scRNAseq/requirements_file/markers.xlsx
#?
```

3.1.4. Choose data format: 'count matrix' write '1' ; 'normalized expression matrix' write '2' ; separate file genes.tsv (list of genes name), names.tsv (list of barcodes or cell names), and sparse matrix (matrix.mtx) write '3' in the console

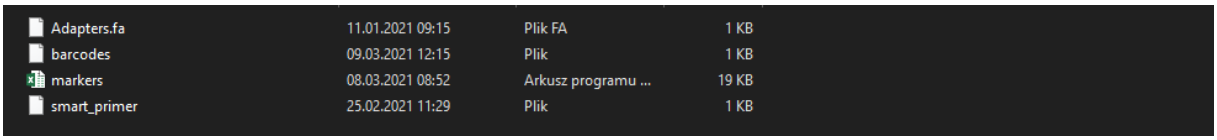
Important: When you use the sparse matrix as input, file names must be for genes - genes.tsv, cell names or barcodes - barcodes.tsv and sparse matrix - matrix.mtx. If not, you will not go to the next step.

If you use count/normalized expression matrix you can give any names, but the matrix must be in .tsv, .csv or .txt format, and then data will be converted to a proper format to build the Seurat object.

3.2. Run analysis

When your project and data is ready you can run the first analysis.

Make sure that used markers are proper for your analysis. Go to JSEQ_scRNAseq/requirements_file/* and check this.



Markers:

Go to file markers.xlsx and check markers. How works cell naming with markers you can read in part

5.10 Cells naming – markers

Counts /expression analysis include:

- Statistical analysis
- Cells naming
- Outlires dropping
- Report generate

Analysis:

3.2.1. Write ‘analysis’ in the console and push enter

```
Choose JSEQ function:
-first installation [install]
-download RefGenome (GENCODE) [genome]
-creat new project [project]
-start analysis [analysis]

Choose function:

analysis
```

3.2.2. To choose ‘counts / expression data’ write ‘2’ in the console

```
analysis
Analysis of data for fastq analysis project [1]  counts / expression analysis project [2]:
```

3.2.3. To choose a project which you want to analyse. Write the project number

```
analysis
Analysis of data for fastq analysis project [1]  counts / expression analysis project [2]:
2
1) /mnt/c/GIT_Projekty/JSEQ_scRNAseq/projects/28-06-2021_test1_exp
2) /mnt/c/GIT_Projekty/JSEQ_scRNAseq/projects/28-06-2021_test2_exp
3) /mnt/c/GIT_Projekty/JSEQ_scRNAseq/projects/28-06-2021_test3_exp
#?
```

It can last a while, some information you can see in the console, but if you want check that all works correctly, go to projects directory ‘project/*project_name*/results/process.log.out’. You can checking and refreshing this log file in real-time during analysis. In the same directory, you can see reads quality control report and statistical single-cell analysis report.

4. Report & Analysis

4.1. Quality control of the reads

For quality control of reads, adapters trimming and repairing were used ‘fastp’ programme, which almost created report. Quality control report you can find in ‘project/*project_name*/results/QC_REPORT’Report contains:

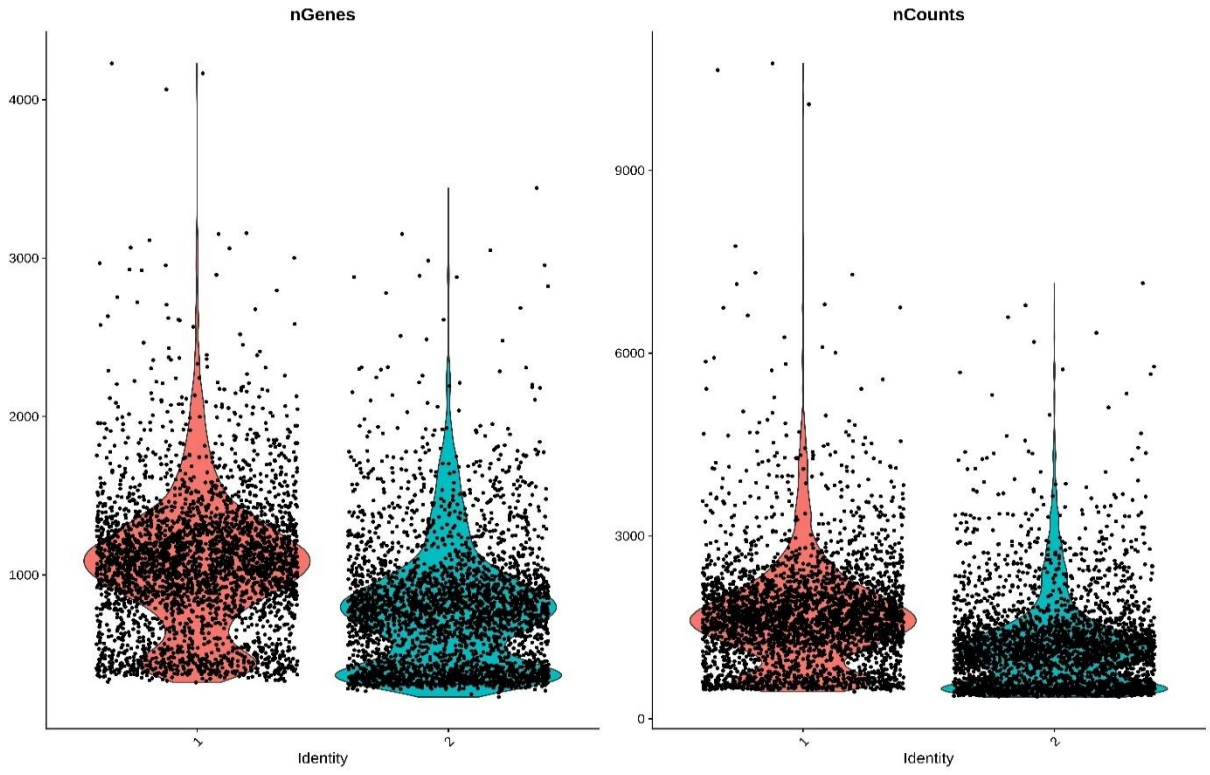
- Adapters content
- Adapters sequence
- Bad ligation adapters
- Nucleotide content graphs

5. Single-cell analysis & statistics

At this stage, many analyses are based on the Seurat library - StjaliLab library with elements that have been programmed by me in R language, such as cell naming algorithms based on markers, selection of the appropriate number of principal components (PCs), visualization of the content of readings, dropping outliers and creating cell subtypes based on markers selected for individual PCA clusters. More information will be provided later.

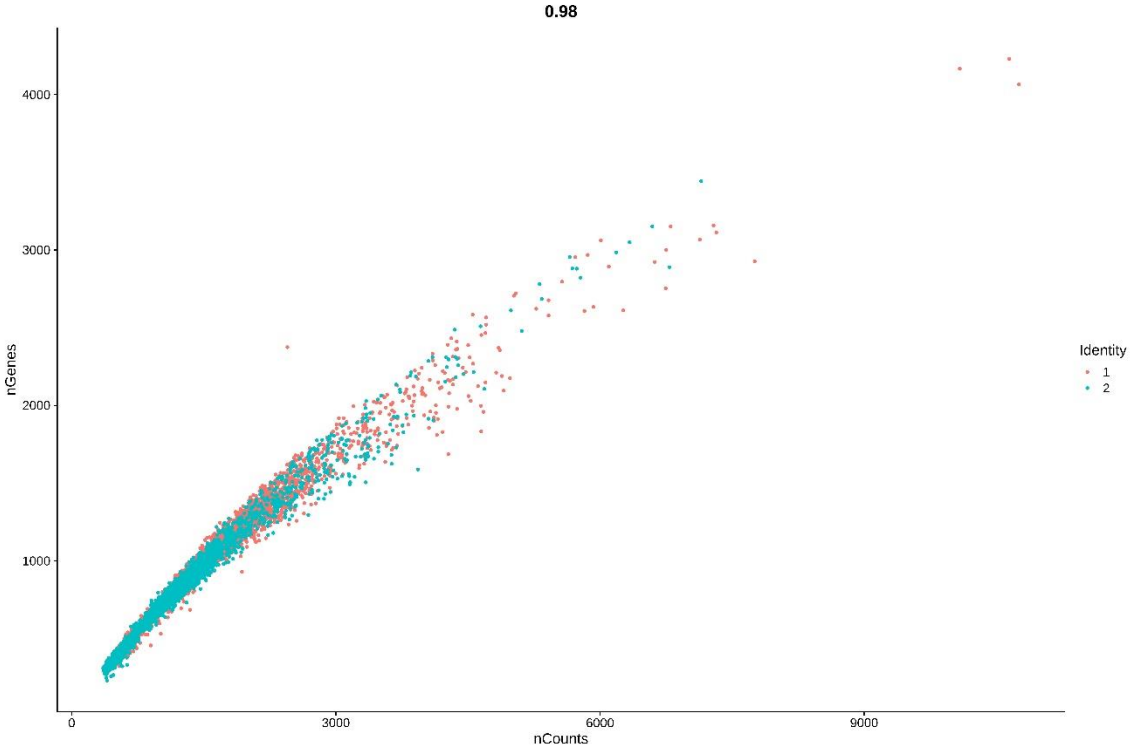
5.1. Cells content information

Counts ~ genes - density plot



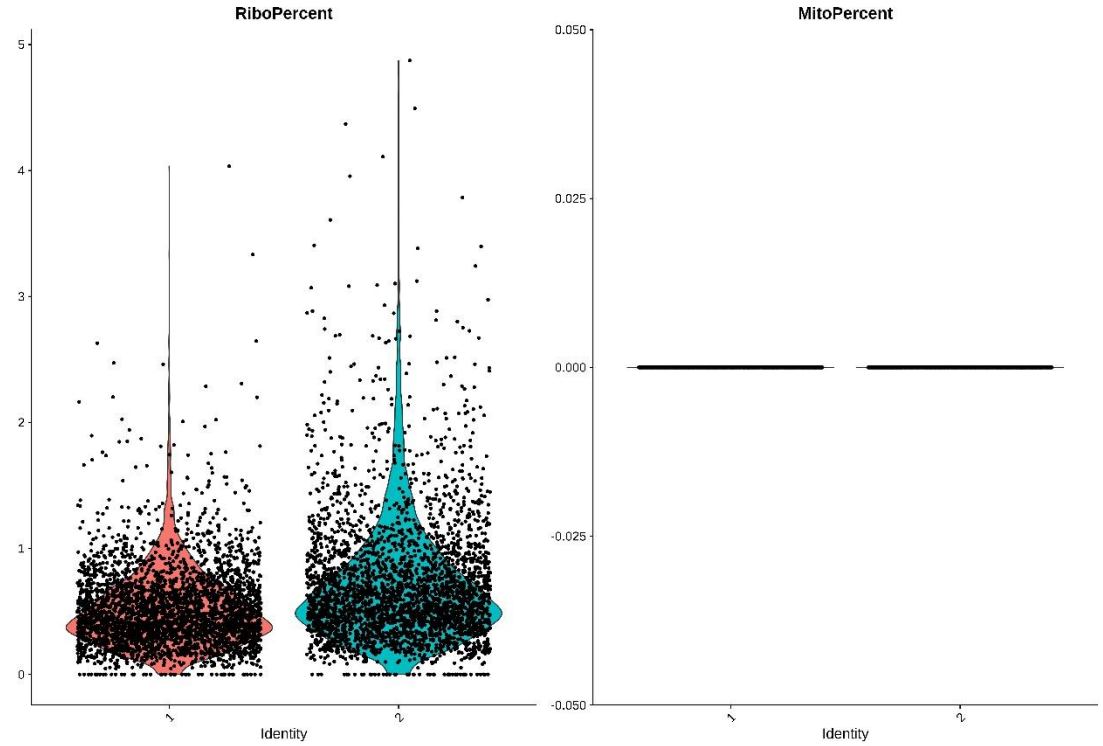
These plots show the density for the number of counts and identified genes for each cell in the analysis.

Counts ~ genes - correlation plot



This plot shows the correlation between the number of counts and identified genes for each cell in analysis.

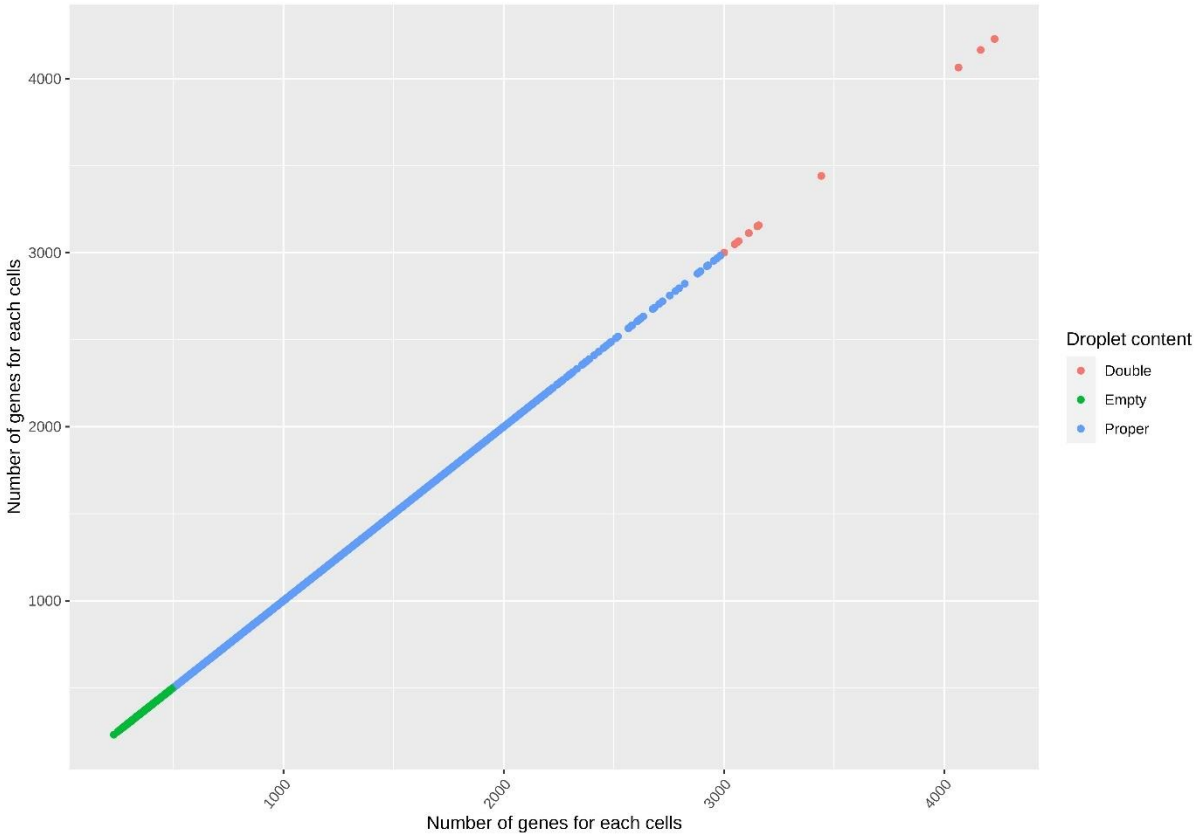
Percent of mitochondrial and ribosomal genes – density plot



These plots show the density of mitochondrial and ribosomal genes in percent scale [%] for each cell

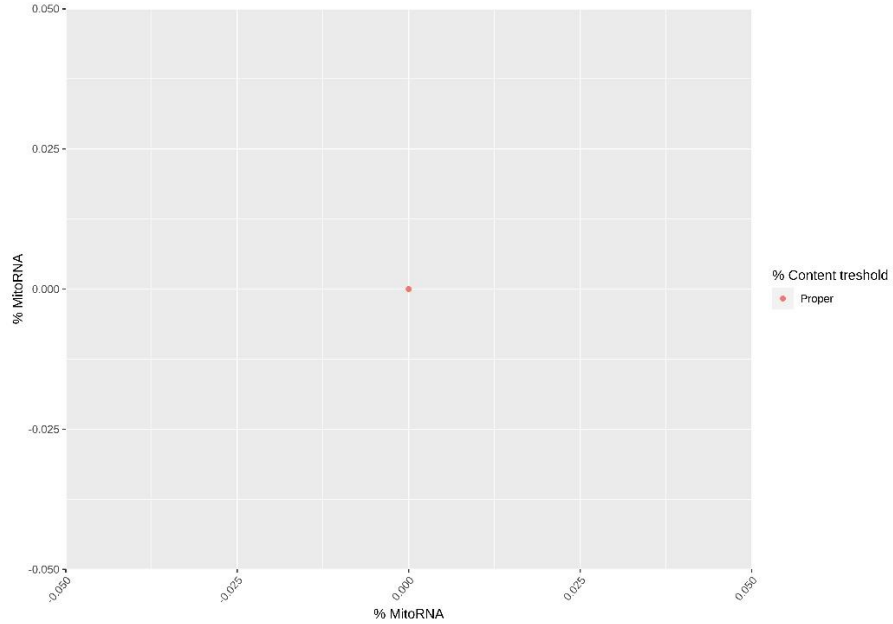
5.2. Outliers and thresholds

Genes number plot



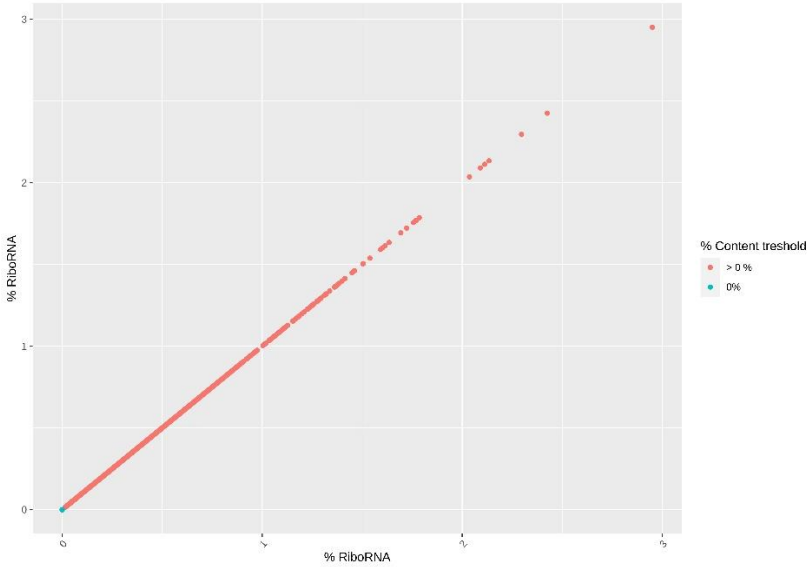
This plot shows compare the number of genes to the number of genes for each cell. Thanks to this we can see outliers values. The threshold is set for bottom values on a minimum of 500 genes per cell and for top values is calculated for most outliers values based on two times mean value plus one and half times the interquartile range (2*MEAN + 1,5*IQR) as the maximum value. Cells below bottom values are recognized as empty cells and cells upper top pom values as doublets, triplets, etc. However, Seurat can deal with doublets but double-check the most outliers cells and removing them can improve further results.

Mitogenes content - plot



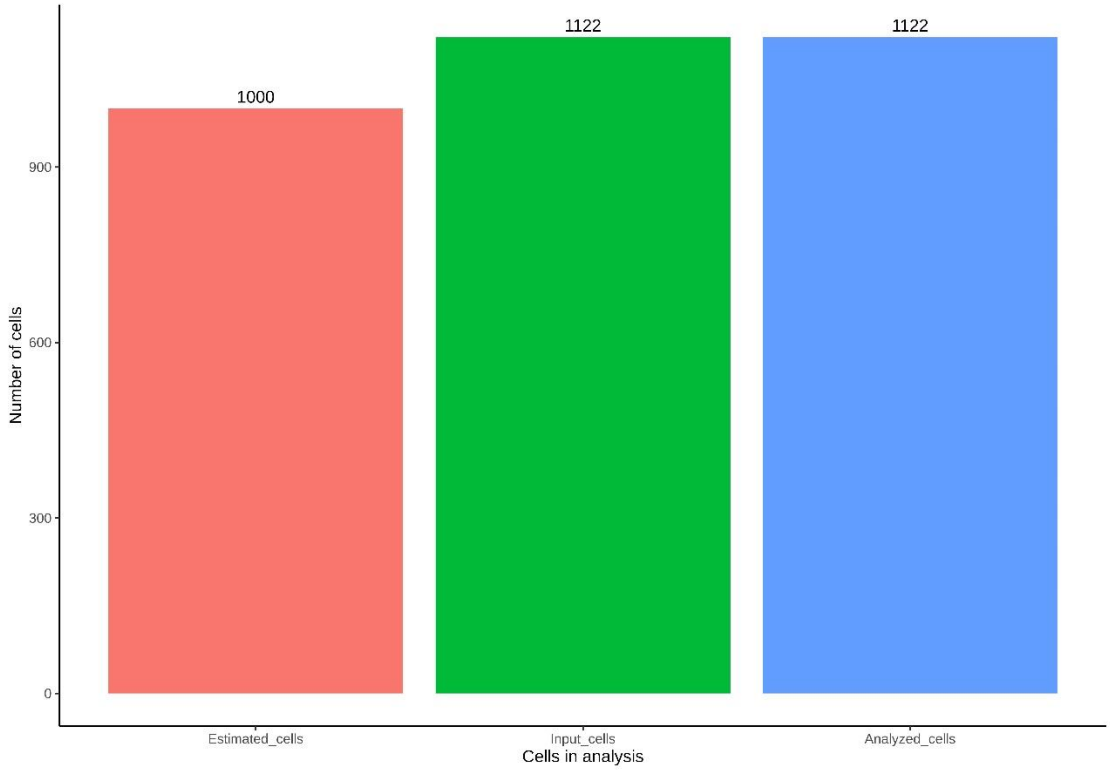
The threshold for mitogenes content was set at 5% of all genes displayed in a single cell. All cells that display mitochondrial genes greater than 5% are removed. In this chart, we can see that all cells have 0% mitogenes. Because these data were obtained by a single nucleus method, mitochondrial "mt-" genes were excluded.

Ribogenes content - plot



For ribogenes there are not set threshold. This plot shows only ribogenes content equal 0% and above 0% in each cell. This knowledge can be used in some experiments to see outliers values.

Amount of the cells in different stages of analysis – plot



This plot shows us estimated amount of cells, cells amount at the beginning of analysis and after quality control with set thresholds.

5.3. Data normalization

The Seurata Normalize data function with "Log Normalize" normalization and the scale factor "1e6" (CPM) was used to normalize the data.

Formula:

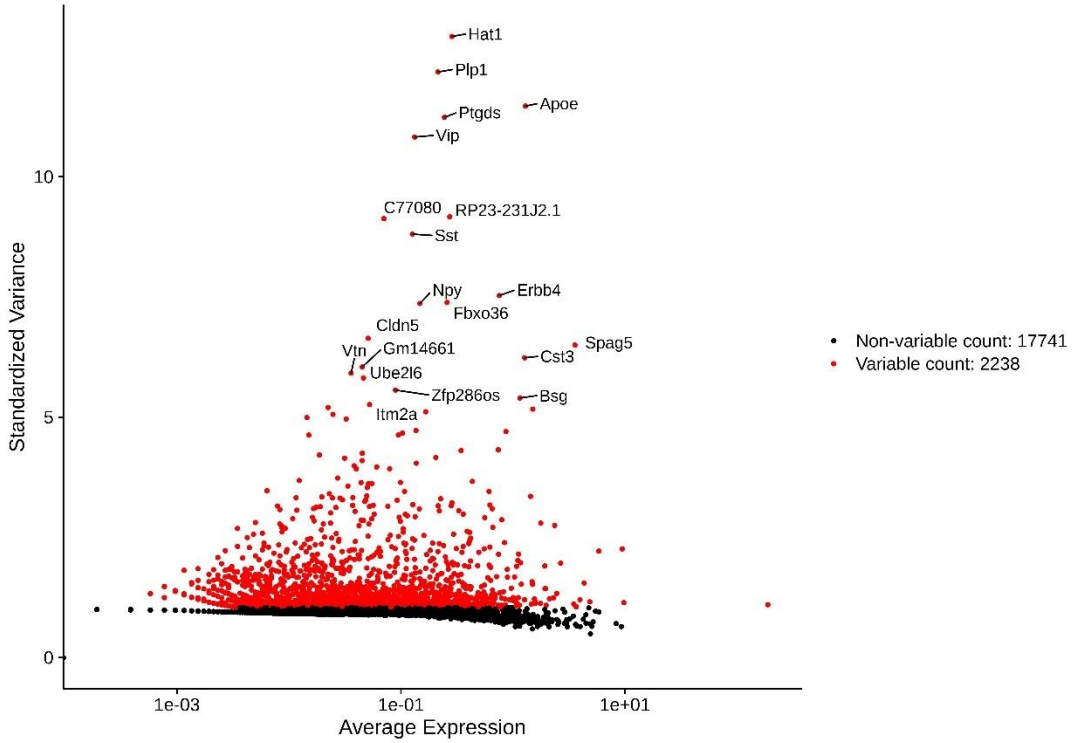
$$CPM = \frac{\frac{\text{counts of gene}}{\text{sum of counts}}}{1000000}$$

$$NormalizedData = \log\left(CPM + 1\right)$$

5.4. Most variable genes

To calculate the most variable genes was used ‘vst’ (Variance Stabilizing Transformation) selection method with ‘equal_frequency’ method based on the Seurat function FindVariableFeatures. The number of genes used by function were estimated as 75% of genes from top expressed cell.

Most variable genes - plot



This plot shows the highly variable genes among all genes from all analysed cells.

5.5. Principle components (PCs)

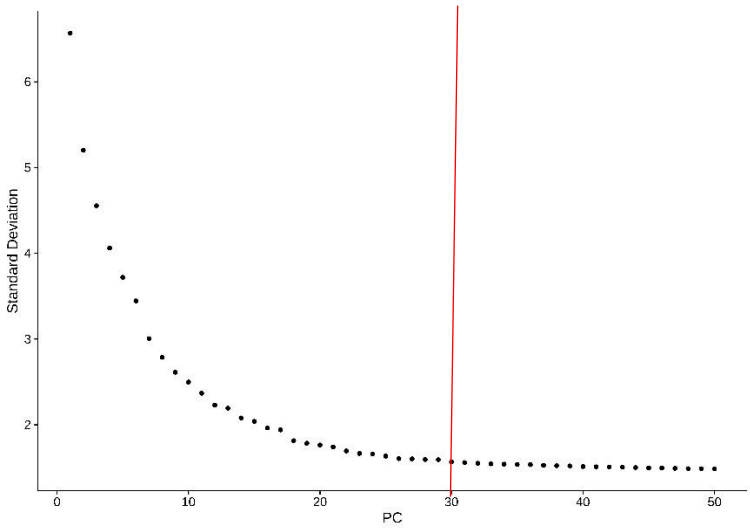
Principle components are a method that is responsible for reducing the dimensionality of ‘p’ numerical variables for each ‘n’ element, increasing interpretability without losing any information (Jolliffe & Cadima, 2016). Thanks to this method we can deal with gene expression matrix for a great number of cells. It is very difficult to compare all genes (p) (for example for human and mice it is about 30 000 genes) in all cells (n), so when we use PC method we can obtain only statistical important information in form of PCs which explain a maximal amount of variance in data set.

Principle components were calculated on scaled data (ScaleData function) with most variable features (genes) using Seurat function ‘RunPCA’.

5.6. Selecting the right number of PCs

To select the right number of PCs for a current dataset in this pipeline, the selection was based on the Elbow plot, JackStraw plot and a special algorithm that checked changes in standard deviation between next PCs. The Elbow plot was created using Seurat functions 'ElbowPlot' with the maximal number of dimension 50.

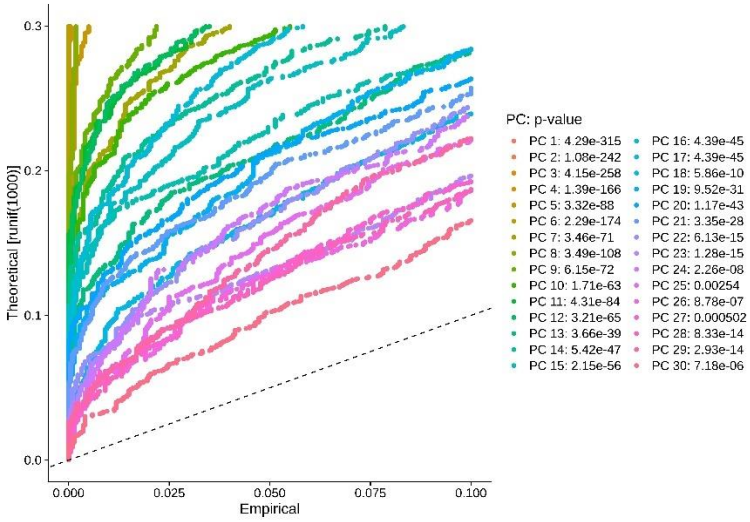
ElbowPlot



In this case, the algorithm chose 30 as the right number of PCs for this data set.

In order to check the correctness of the selected number of PCs, the JackStraw function was used to indicate the statistical significance of individual PCs.

JackStraw plot



Here we can see a p-value for each PCs and if it is statistically significant.

5.7. Data clustering

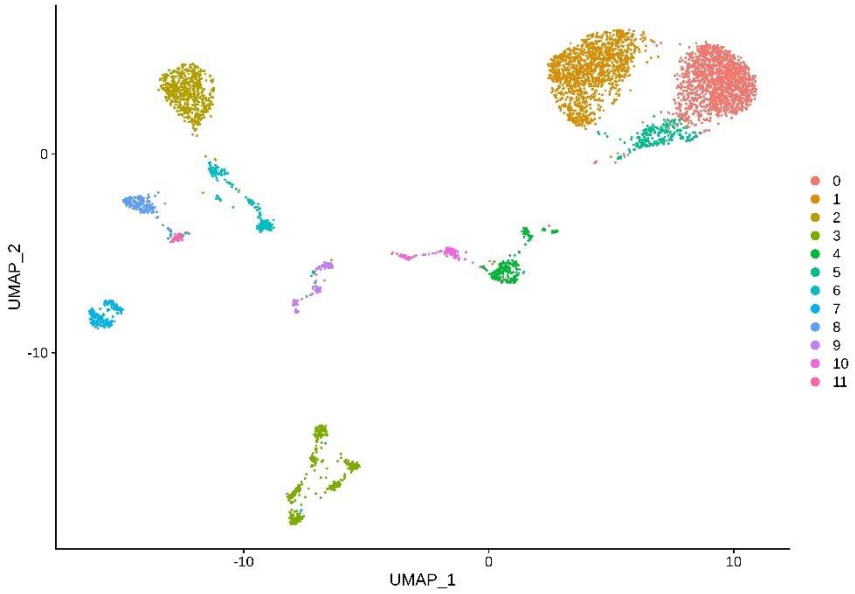
For data clustering based on previously selected PCs were used two Seurat functions: 'FindNeighbors' and 'FindClusters'. FindNeighbours function is based on kNN (k-nearest neighbour) algorithm. This function return kNN information for whole datasets based on PCs. Default parameter k in this pipeline for the KNN algorithm is set on 49. FindCluster function based on kNN results identify cells clusters by a shared nearest neighbour (sNN) algorithm. FindCluster function is set on resolution 0.5, the number of starts 10 and the number of iterations 1000.

Both used algorithms are common for single-cell analysis and provide obtain clusters that are connected with different cells populations (Zhu et al., 2020). In the next steps based on these clusters, we obtain marker genes for each cluster, name cells populations using known gene markers and divide cell populations into cell subtypes.

5.8. UMAP - Uniform Manifold Approximation and Projection for Dimension Reduction

In contrast to traditional linear dimensionality reduction method like PCA is non-linear UMAP. The UMAP method is another method based on dimensionality reduction and is similar to tSNE method belongs also to the non-linear visualization method. The main role for UMAP algorithms was single-cell data visualization (Narayan et al., 2020), so it was the reason why there were choose it for data visualization in this pipeline. What is more, UMAP seems to be more convenient than tSNE method, which can be problematic with large data sets. UMAP optimize the embedding coordinates of individual data points using iterative algorithms and constructs a high dimensional graph representation of the data then optimizes a low-dimensional graph to be as structurally similar as possible.

UMAP plot



In this step, was obtain the UMAP plot with signed clusters (numeric factors) obtained via KNN / SNN algorithms from previous steps.

5.9. Cluster markers

To obtain specific markers for clusters were used Seurat function 'FindAllMarkers'. As the selecting method used algorithm 'MAST' with only positive markers and with the minimum frequency of markers in cluster 0.25. MAST algorithm for single-cell gene expression is based on two-part generalized linear models (GLM). First part models the discrete expression rate of each gene across cells, and the second part models the conditional continuous expression level (Finak et al., 2015).

MAST markers

```
##            p_val avg_logFC pct.1 pct.2     p_val_adj cluster          gene
## 1   0.000000e+00 1.7252321 0.678 0.240  0.000000e+00       0       Gm12027
## 2   0.000000e+00 1.5150667 0.857 0.422  0.000000e+00       0          Akt3
## 3   0.000000e+00 1.4701830 0.992 0.594  0.000000e+00       0         Pde1c
## 4  2.487357e-160 1.6234647 0.414 0.126 4.969490e-156       0       Gm8257
## 5  8.480706e-134 2.1502626 0.267 0.049 1.694360e-129       0       Gm31925
## 6  3.431790e-130 0.9527619 0.657 0.280 6.856374e-126       1         C1ql3
## 7  2.919557e-120 1.0356293 0.588 0.230 5.832983e-116       1          Dgkh
## 8  4.785292e-101 1.1191092 0.489 0.189  9.560535e-97       1        Lman2l
## 9   1.614001e-97 0.9957944 0.507 0.196  3.224613e-93       1         Tanc1
## 10  8.523628e-69 1.5826682 0.271 0.084  1.702936e-64       1          Kri1
## 11  0.000000e+00 3.0471364 0.937 0.070  0.000000e+00       2       Gm28928
## 12 3.418833e-240 2.7580509 0.607 0.052 6.830486e-236       2        Pdzrn3
## 13 4.567684e-168 2.4996415 0.453 0.035 9.125776e-164       2         Vwc2l
## 14 3.283246e-141 3.0890368 0.374 0.033 6.559597e-137       2 2810459M11Rik
## 15  1.263893e-93 2.5857361 0.261 0.018  2.525133e-89       2          Cux2
## 16  0.000000e+00 3.9607792 0.900 0.103  0.000000e+00       3         Erbb4
## 17 3.074380e-191 4.1234410 0.488 0.011 6.142304e-187       3          Gad2
## 18 8.603883e-174 4.6134760 0.420 0.006 1.718970e-169       3       Dlx6os1
## 19 2.778522e-108 3.9512850 0.292 0.006 5.551210e-104       3        Dlx1as
## 20 8.807278e-108 4.0559266 0.332 0.019 1.759606e-103       3           Npy
```

Obtained markers are helpful to determine to which a cell population belong cluster.

Moreover, these markers will use for dividing subclass clusters into cell subtypes based on the Cell Subtypes Selection by Genes (CSSG) algorithm.

5.10. Cells naming - markers

For cells naming were wrote special Cell Cluster Naming (CCN) algorithm, which checking the most expressed marker for each cluster. There are two approaches for cluster naming. The first approach based on known, defined by user markers.

User markers are in the excel file in JSEQ_scRNAseq/requirements_file/markers.xlsx. There we have two types of markers: the first type is in the first sheet (cell class) and the second type is in the second sheet (cell subclass).

Cell classes markers

Astrocytes	Oligodendrocytes	Microglial	Endothelial	OPC	Ependymal	Macrophag	Pericytes	Purkinji	Tanacytes	Fibroblast	Granule	Intermediate progenitors	Neuroepithelial
+AQP4	+OPALIN	+TYROBP	+NOSTRIN	+PDGFRA	+FOXJ1	+MRC1	+VTN	+PPP1R17	+RAX	+DCN	+GABRA6	+PAX6	+PAX2
+GFAP	+MOG	+C1QC	+CLDN5	+CSPG4		+CX3CR1							
+SLC1A3	+PTGDS												
	+OLIG2												
	+OLIG1												

You can change your markers depending on yours data and experiments, but remember if you write your own markers for cell classes you have to add '+' before the marker gene name. Gene markers without '+' will not readable. Is a good manner to save markers without using them in analysis.

Cell subclasses markers

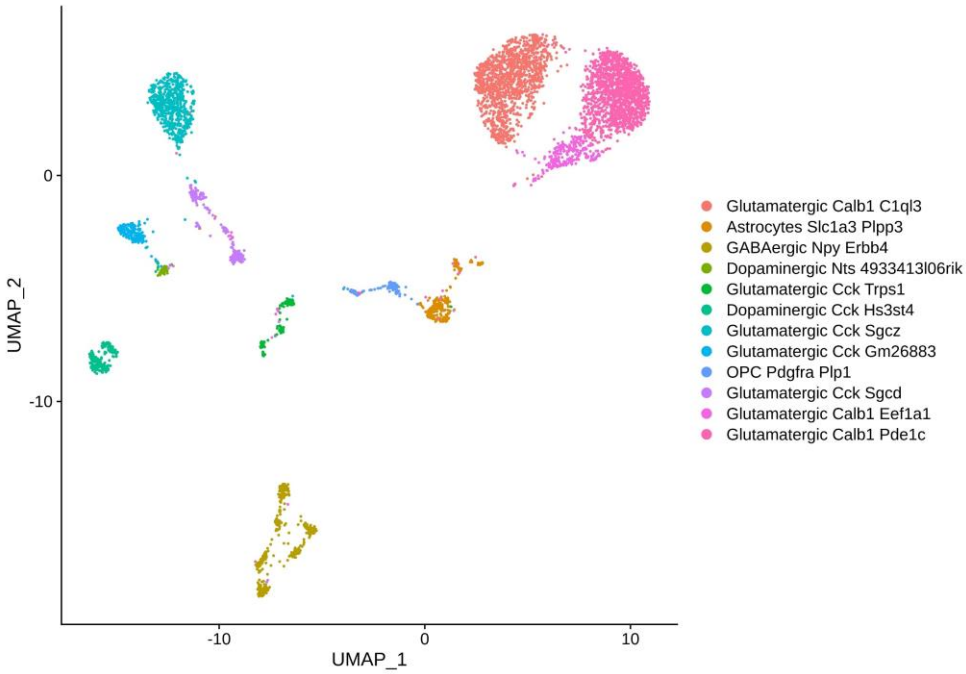
VIP	SST	PVALB	CCK	GAL	CARTPT	NMU	TAC1	NTS	AVP	OXT	GNRH1	PENK	HCRT	TRH	POMC	AGRP	NPY	NMU

In this case, you need not use additional '+' for markers.

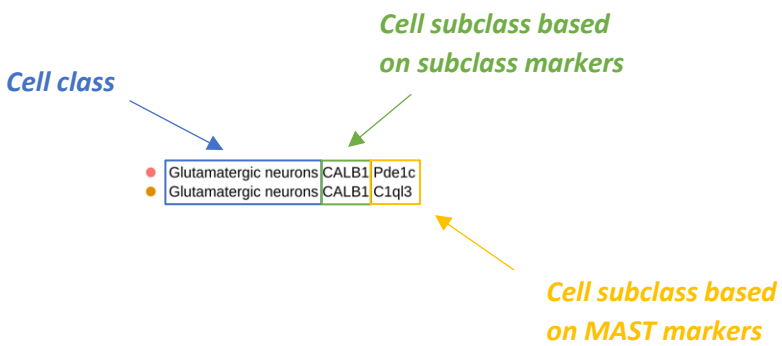
The presented set of markers include markers that determined non-neuronal and neuronal cells class and additionally markers for neurotransmitters, neuropeptides, their receptors and calcium-binding proteins as genes involved in interneuronal signalling. Both sets of markers are based on a great number of publications(Anderson et al., 2020; Artegiani et al., 2017; Carter et al., 2018; Chen et al., 2017; Ehman et al., 2017; Eze et al., 2020; Fan et al., 2020; Gokce et al., 2016; Guo & Li, 2019, 2019; Habib et al., 2017; Harris et al., 2017; Kalish et al., 2018; Keo et al., 2017; Kishimoto et al., 2018; Koirala & Corfas, 2010; Kozareva et al., 2020; Langlet, 2019; Martínez-Cerdeño & Noctor, 2018; McKenzie et al., 2018; Mickelsen et al., 2020; Muñoz-Manchado et al., 2018; Nelson et al., 2020; Peng et al., 2019; Smith et al., 2019; Takeuchi et al., 2020, 2020; Zeisel et al., 2018).

The first naming manner is based on three types of markers: class markers, subclass markers and cluster-specific markers selected by MAST. In the first step, the algorithm checks the cell class based on the most expressed class marker (log(CPM+1)) and give the name to the class. In the next steps, the algorithm, in the same way, giving names for cell subclass and then subtypes.

Cells naming scheme – first manner

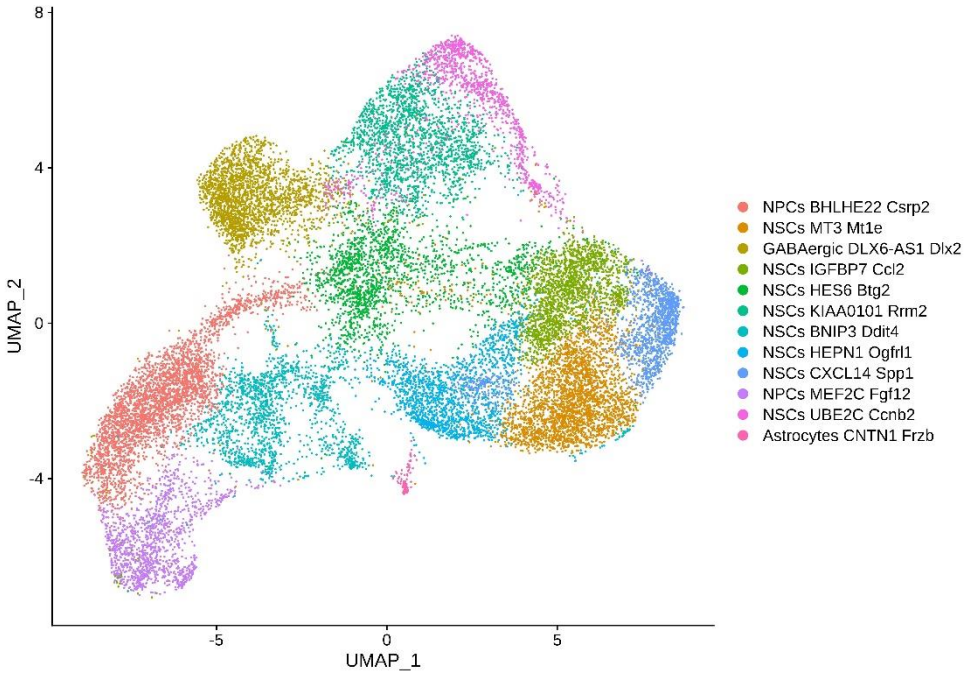


In this plot, are presented cell classes and subclasses after naming. Below is showed scheme of the cell name.

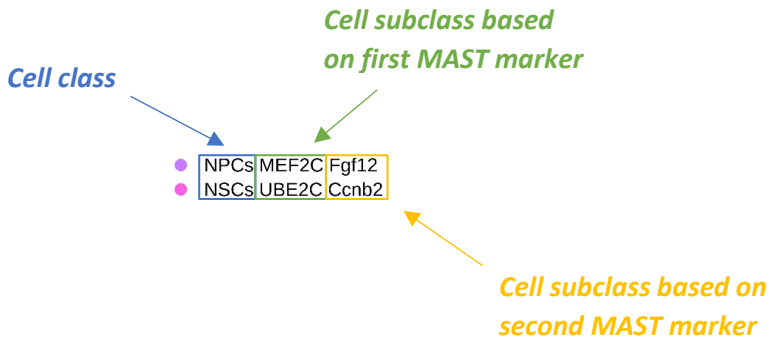


The second naming manner is based on two types of markers: class markers and selected by MAST markers. In the first step, the algorithm checks the cell class based on the most expressed class marker (log(CPM+1)) and giving the class name. In the next steps, the algorithm checks the cell subclass based on the first and second most expressed MAST typed markers, and giving the subclasses names.

Cells naming scheme – second manner



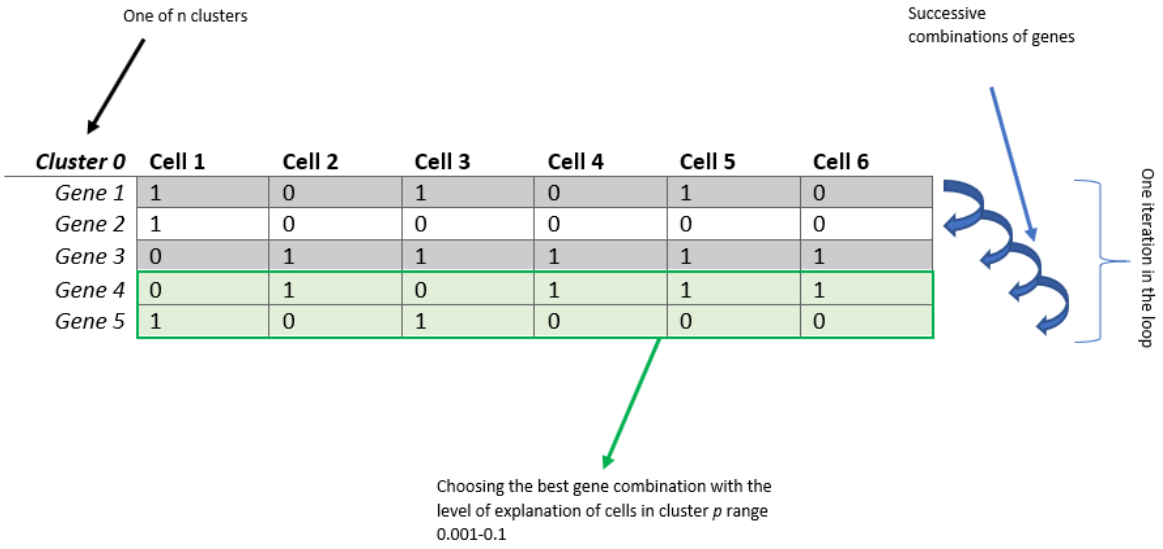
In this plot, are presented cell classes and subclasses after naming. Below is showed scheme of the cell name.



5.11. Dividing the cells subclasses (clusters) into cells subtypes

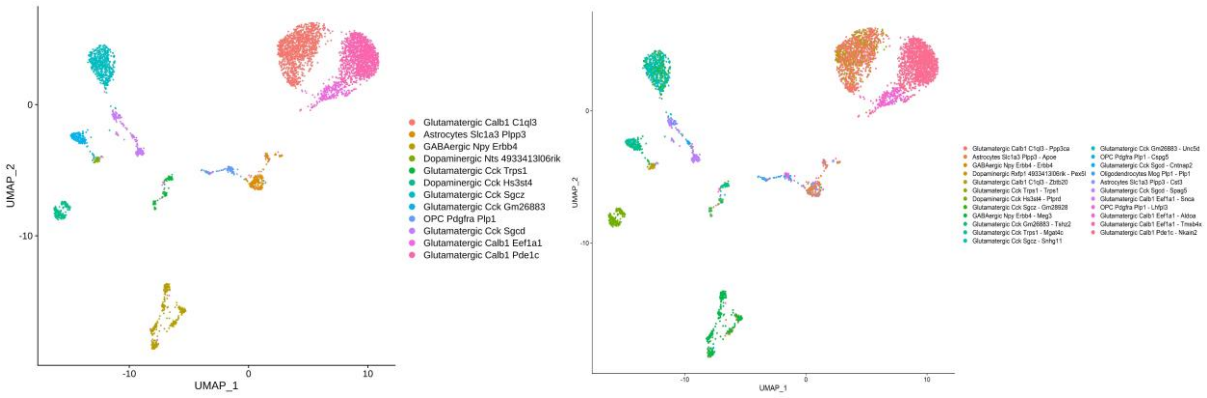
Due to high heterogeneity inside clusters obtained in the basic single-cell analysis were written Cell Subtypes Selection by Genes (CSSG) algorithm. The CSSG algorithm is based on markers selected separately for each cluster by the MAST algorithm. The algorithm creates a binary matrix where 0 means no expressed genes and 1 means genes with an expression not equal to 0. Then, for each cluster, the best combinations of genes are selected in successive iterations, explaining the largest possible number of cell subtypes with the use of the smallest possible number of genes at the level of significance in the range p = 0.001-0.1.

Example of CSSG algorithm iteration for dominant genes selection in the cell subtypes



Cluster 0	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6
Gene 1	1	0	1	0	1	0
Gene 2	1	0	0	0	0	0
Gene 3	0	1	1	1	1	1
Gene 4	0	1	0	1	1	1
Gene 5	1	0	1	0	0	0

Data presentation with and without CSSG algorithm

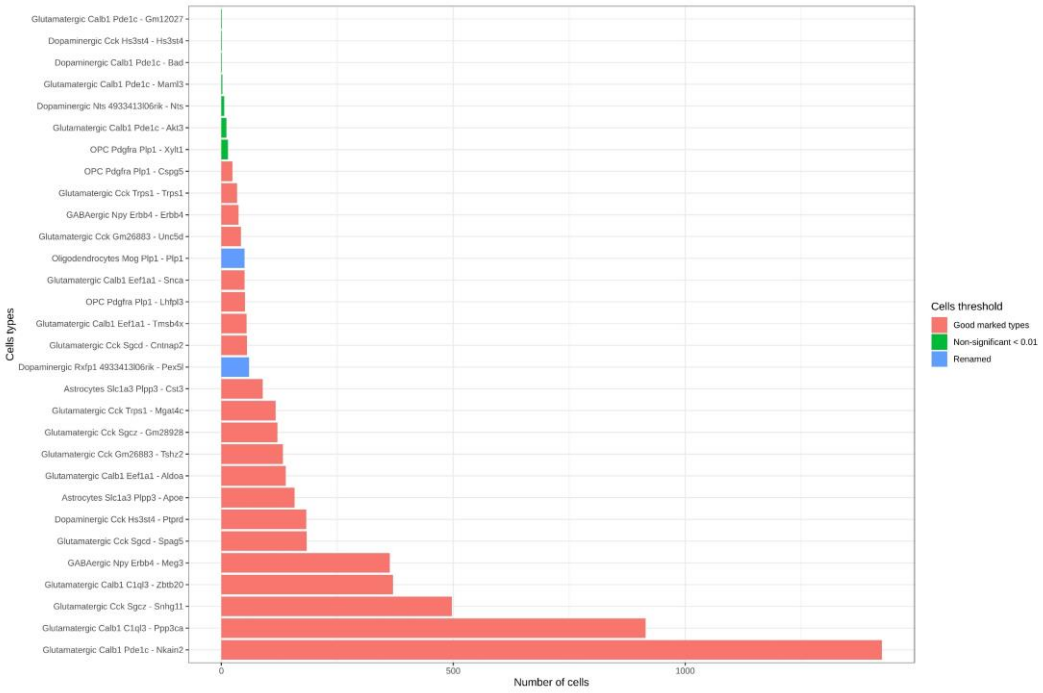


The JSEQ® CSSG algorithm was tested on 812 945 single cell from different brain regions. Results after using the JSEQ® CSSG algorithm shows that the algorithm can improve the resolution of obtained results. Based on the obtained result we can see that algorithm allow:

- split cells subclasses into two different subclasses (e.g. from oligodendrocytes to oligodendrocytes and OPCs) or subtypes with different development stage,
- based on dominant marker shows a difference in cell's maturation stage,
- shows different status of obtained cell subtypes e.g. based on signalling peptides secretion in neuronal cells.

5.12. Outliers dropping

This pipeline contains a lot of checkpoints that protect against bad quality or badly clustered cells. Despite duplicates removing at the beginning and additional selection points in the pipeline was projected another quality control step. After dividing cells populations with the CSSG algorithm, obtained cells subtypes in new clusters are checked in terms of proper names. Cell subtypes groups that were badly marked or their names were changed are renamed to the right form. Furthermore, when new cell subtypes do not express markers selected by CSSG, then they are drop out from the analysis. Also, cells subtypes whose amount is lower than 0.01 amount of the largest subtype in the analysis are dropped.

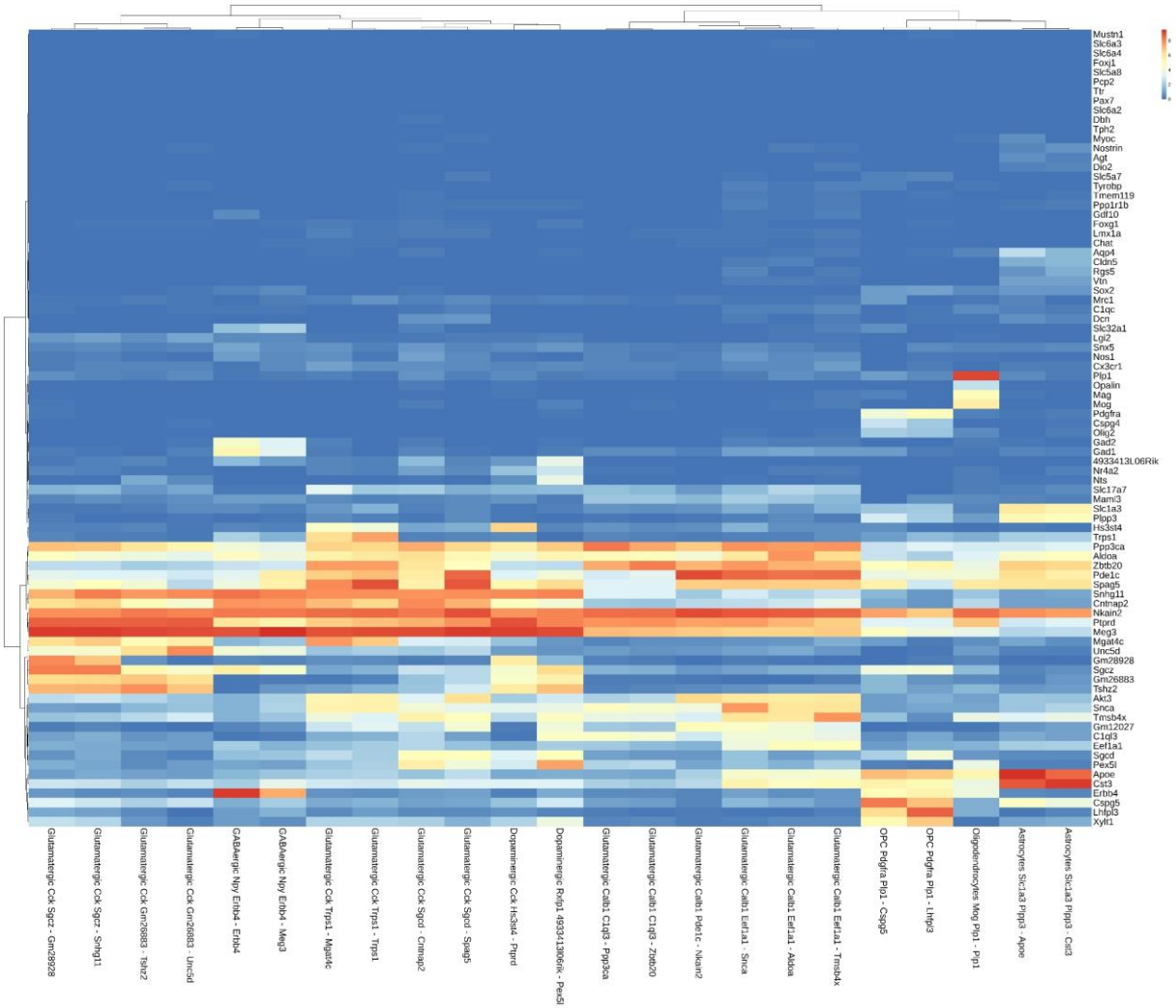


This plot shows how many cells were good marked, bad marked, renamed and non-significant.

All dropped cells from the analysis are saved to a new dataset in repository: 'JSEQ_scRNAseq/projects/*project_name*/results/exp_matrix/unknow_cells_count_matrix.xlsx'

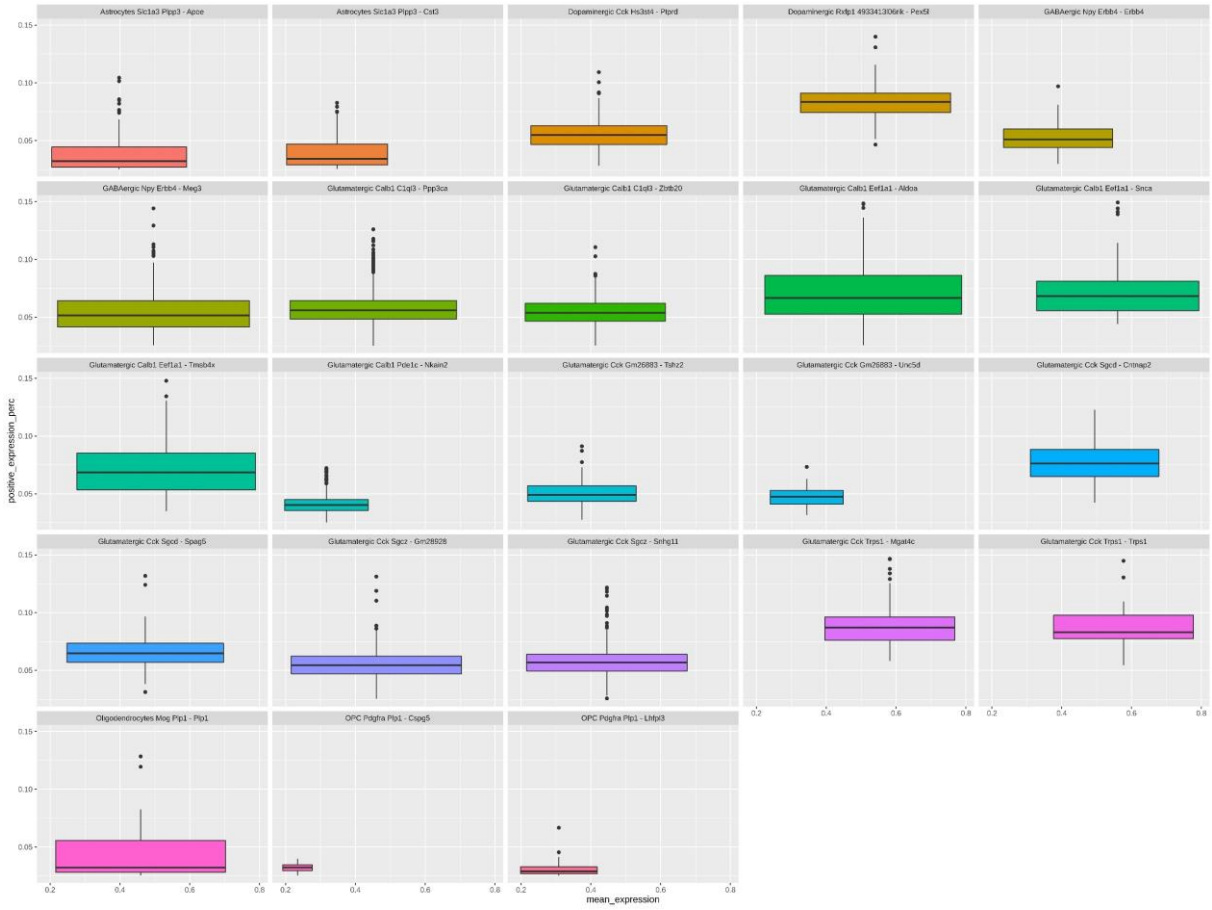
5.13. Cells subtypes visualization

Pheatmap



This plot shows all obtained cell subtypes with used markers for class names, subclass names and selected with the MAST algorithm.

Boxplots for genes ratio and level expression



It is the last plot in the analysis which shows on the x-axis percent of expressed genes for all cells included in this cell subtype and on the y-axis mean expression value for all cell in this subtype. Thanks to this plot we can check the heterogeneity/homogeneity obtained in analysis cell subtypes.

6. References:

Graphics:

Biorender

https://biorender.com/

Used software and scripts:

DropSeqPipe

https://github.com/Hoohm/dropSeqPipe

STAR

https://github.com/alexdobin/STAR

Drop-seq

https://github.com/broadinstitute/Drop-seq

Picard

https://github.com/broadinstitute/picard

fastp

https://github.com/OpenGene/fastp

Seurat

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