JSEQ® manual

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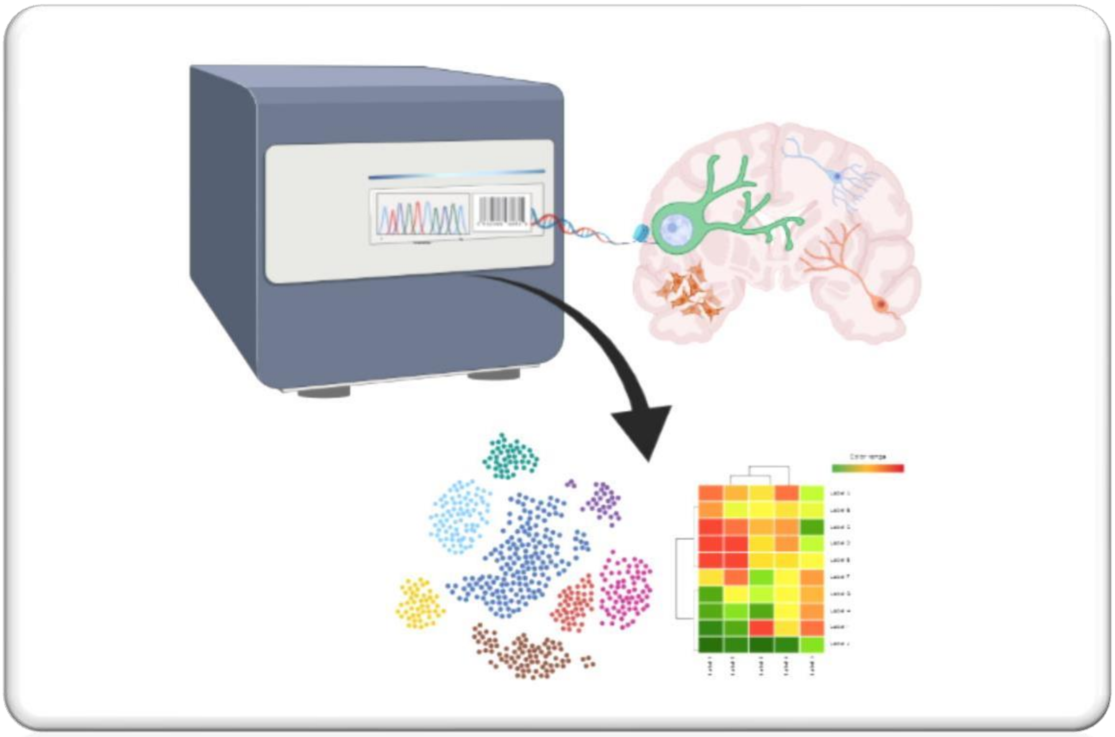
JSEQ® - single cell sequencing analysis tools



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Introduction

Single-cell sequencing is a modern technique of sequencing at the single-cell level. Such an approach gives many possibilities to get knowledge about gene expression in different cell populations during development, carcinogenesis, or after treatment with novel gen therapy. However, how does it work? So-called single-cell sequencing does not focus on 'single cell' but on whole populations of cells we sequence. So whence do we obtain information about a single cell? Currently, we have some approaches for single-cell sequencing, but the final effect is similar. Single-cell sequencing results in a significant number of cell transcriptome sequences with unique sequence signs such as 'barcode' and 'UMI' - Unique Molecular Identifiers. The barcode sequence is different for each cell during sequencing. Besides, 'UMI' is independent of the barcode sequence, which allows distinguishing each copy of mRNA (count) in the cell (for each barcode) and removing duplicate values. So there are two essential 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 we have thousands of cells' transcriptomes during sequencing and analysis. After sequencing, obtained results are conducted by multi-stage quality control and statistical analysis (e.g., variable reduction by PCA, clustering algorithm 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 input types.

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1. First use and installation

The JSEQ® pipeline was prepared for LINUX systems, so if you are a WINDOWS user and you have Windows 10 or 11, do not worry. Currently, Microsoft® offers a good LINUX subsystem without changing the basic WINDOWS system. So if you still do not have the LINUX subsystem on your PC, go to Microsoft Store, install Ubuntu, and enjoy your first steps in the LINUX environment; and if your Ubuntu system is ready, download JSEQ®.

1.1. Working directory

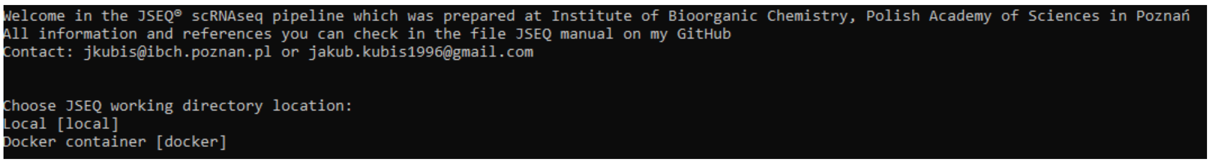
In the JSEQ® 2.1.1 release, an option was added to choose where the pipeline required tools should be installed. If you choose 'local' then the whole pipeline will be installed at localhost, or when you choose 'docker' the requirements will be installed in the docker container (before using this option, 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 correct 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 the program by writing './JSEQ' in the console

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

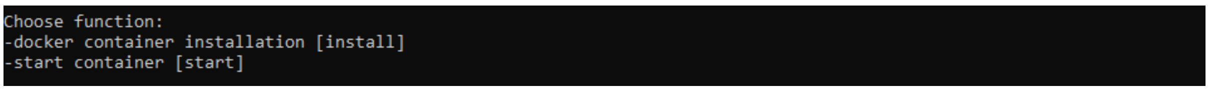


```
Welcome in the JSEQ® scRNAseq pipeline which was prepared at Institute of Bioorganic Chemistry, Polish Academy of Sciences in Poznań
All information and references you can check in the file JSEQ manual on my GitHub
Contact: jkubis@ibch.poznan.pl or jakub.kubis1996@gmail.com


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

1.2. Installation

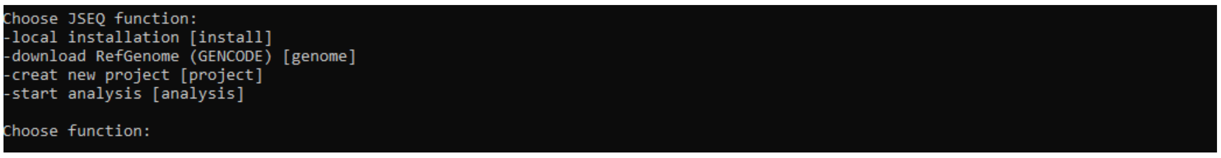
1.2.1. Write the command 'install' in the console and push enter

Docker container:



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

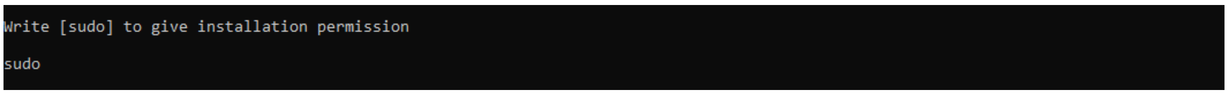
Localhost:



```
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 that give JSEQ pipeline.**

1.2.2. In the case of localhost, write 'sudo' to permit installing all the required files



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

1.2.3. Wait for the installation to 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 the results

1.3. First use

If the JSEQ were installed, you could go to the rest options of the pipeline. Each of them was described in the following chapters, where you can find how they work and how to use them.

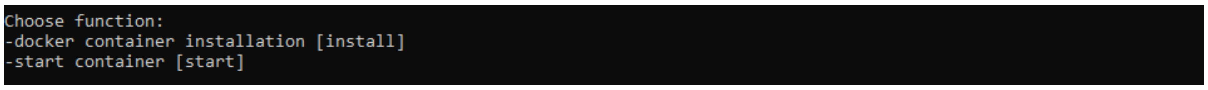
I want to point out that JSEQ can be used for analysis at different steps. The first option gives the possibility to start the analysis from fastq files. First, you should choose the option' download RefGenome' when you want to run it. This option will download a reference genome and gene annotations from the https://www.gencodegenes.org/ website. In JSEQ 2.3.1 was added an option for using custom genomes from different sources: ENSEMBLE, NCBI, GENECODE. If you want to use a custom genome for other species, you should go to JSEQ_scRNAseq/requirements_file/genome.conf and change the links in the custom genome config file position. Another option is to run an analysis when we have results after mapping raw reads to the genome. It can be data in the matrix format (or sparse matrix) for raw counts or normalized expression values.

However, you must create a project before running both types of analysis. To do this, you have to run the option 'create new project'. There are two options to choose from, depending on which analysis you choose: raw data (fastq files) or after mapping. The same will regard to 'start analysis' option.

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).

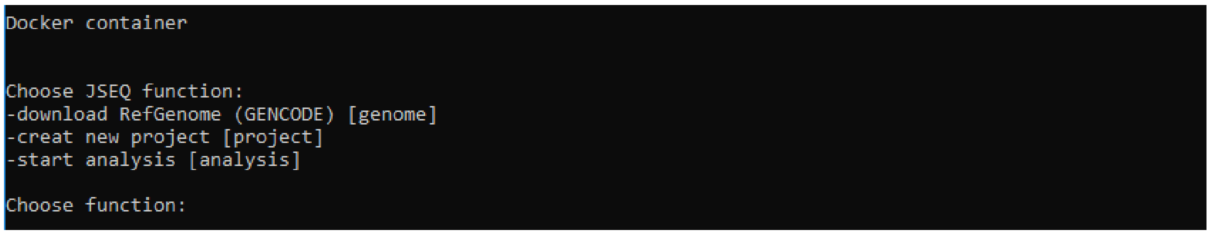
1.4. Working in docker:

1.4.1. Run docker container: write 'start' in console



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

Since then, all the rest functions will be the same as those written below for the local working directory. What is more, results are saved in the local repositories. You can also modify requirements files like markers and all configuration files in the local repository.



```
Docker container


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

Choose function:
```

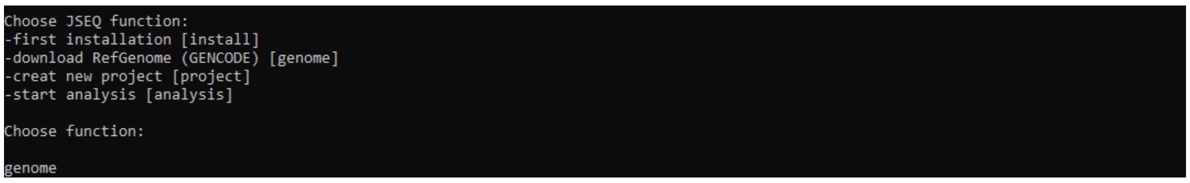
2. Analysis – raw data (fastq files)

2.1. Genome download

Before starting analysis, you must download reference genome and gene annotations properly for your data sets. The downloaded genome will be deposited in a separate directory from your analysis directory. Moreover, after that process of genome annotation will be conducted, but this step will run during project creation because gene annotation depends on your sequenced read length. This step can last a while, so to prevent lost time each time when you create a new project, the annotated genome is also saved in a different directory than your project directory. The gene annotations step will be omitted when you run an analysis in the future with the same length of the reads.

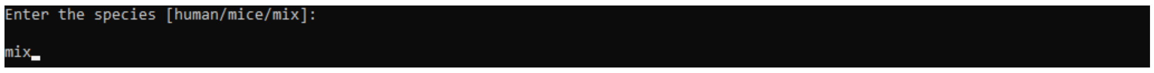
Genome downloading:

2.1.1. Write 'genome' 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:

genome
```

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



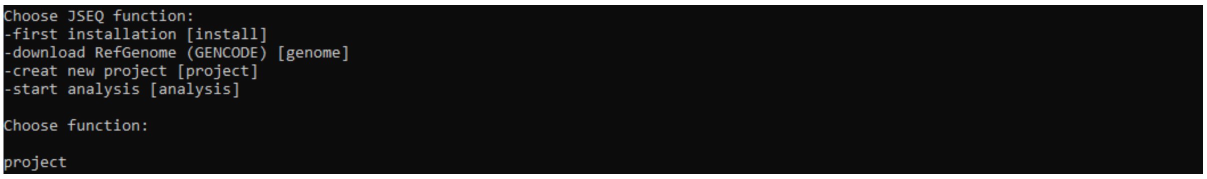
```
Enter the species [human/mice/mix]:

mix
```

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 run the annotation process, which depends on your length.

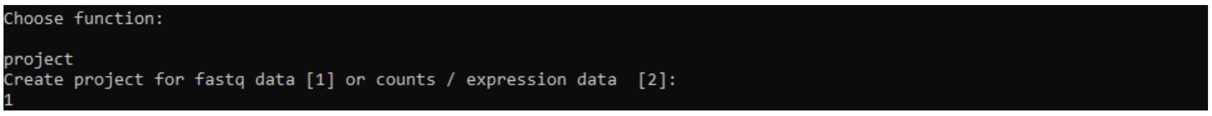
Project creating:

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
```

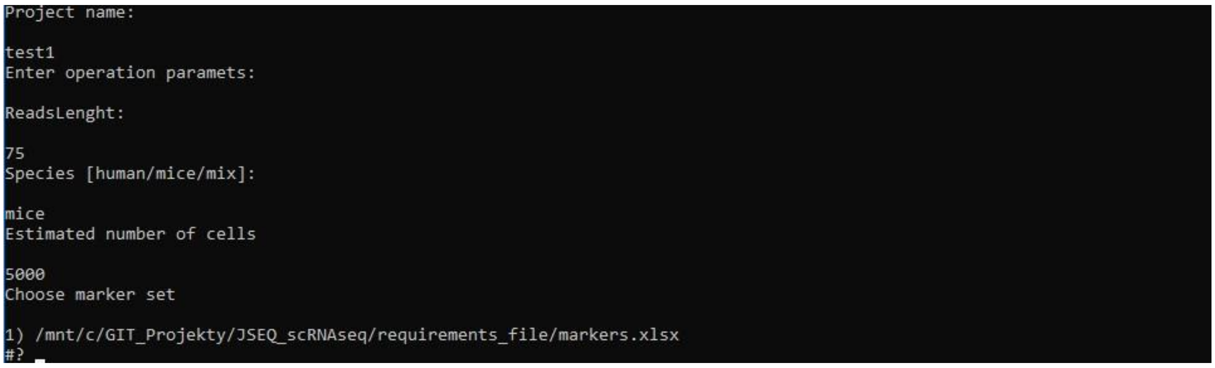
2.2.3. Now you have to fill in some information such as project name, read length, species, the estimated number of cells

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

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

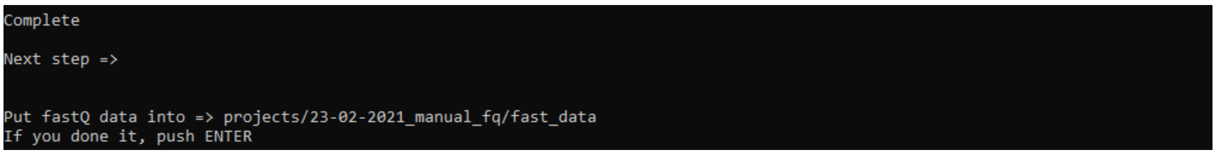
Estimated number of cells – write the number of cells how you expect with your single-cell technology (e.g., dropSEQ).

Marker set – choose the right markers set, which will be used in the current 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 are correct, you will see the message and source where you should put your fastq data. It can be multiple data that will be combined (e.g., 5x Read1_R1.fq and 5x Read2_R2.fq). Each read should provide information about the number of the read in the format of suffix: '_R1'; '_R2'.



```
Complete

Next step =>


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

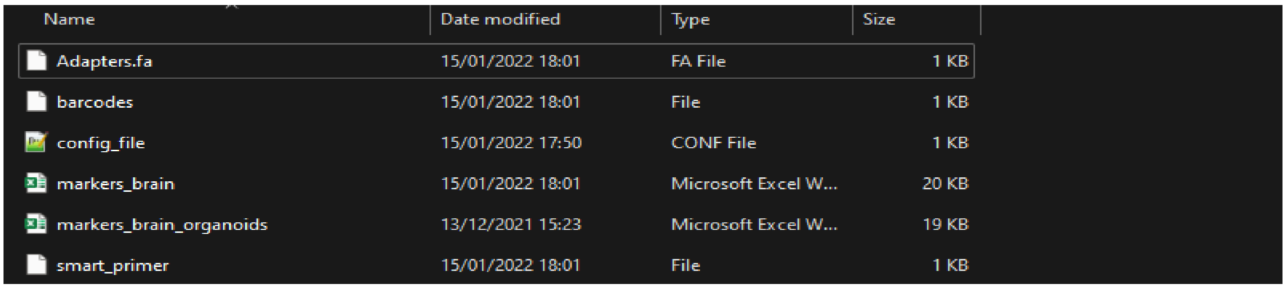
2.2.4. Put data into the given source directory

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

2.3. Run analysis

You can run the first analysis when your project and data are ready.

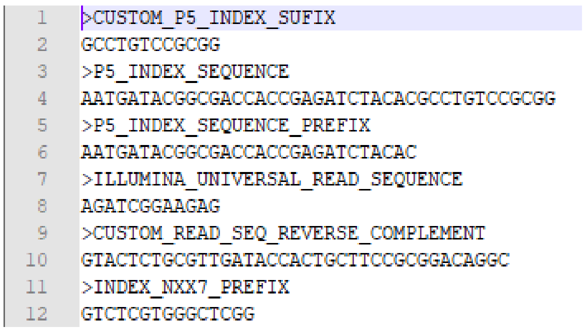
Ensure that used markers, adapters, smart primer sequence, barcode, and UMI length are proper for your analysis [Nadia Dolomit, 10x, etc.]. Go to JSEQ_scRNAseq/requirements_file/* and check this.



Name	Date modified	Type	Size
Adapters.fa	15/01/2022 18:01	FA File	1 KB
barcodes	15/01/2022 18:01	File	1 KB
config_file	15/01/2022 17:50	CONF File	1 KB
markers_brain	15/01/2022 18:01	Microsoft Excel W...	20 KB
markers_brain_organoids	13/12/2021 15:23	Microsoft Excel W...	19 KB
smart_primer	15/01/2022 18:01	File	1 KB

Adapters:

Open the adapters file and check the adapter sequences.

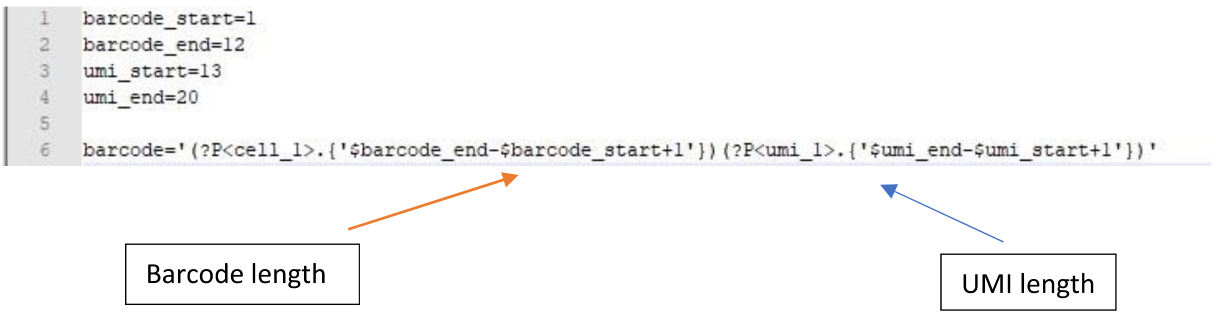


```
>CUSTOM_P5_INDEX_SUFIX
GCCTGTCCGCGG
>P5_INDEX_SEQUENCE
AATGATACGGCGACCACCGAGATCTACACGCCTGTCCGCGG
>P5_INDEX_SEQUENCE_PREFIX
AATGATACGGCGACCACCGAGATCTACAC
>ILLUMINA_UNIVERSAL_READ_SEQUENCE
AGATCGGAAGAG
>CUSTOM_READ_SEQ_REVERSE_COMPLEMENT
GTACTCTGCGTTGATACCACTGCTTCCGCGGACAGGC
>INDEX_NXX7_PREFIX
GTCTCGTGGGCTCGG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

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

Barcodes:

Open the barcodes file and check the 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'})'
```

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

Smart primer:

Go to the smart_primer file and check the primer sequence

```
smart=AAGCAGTGGTATCAACGCAGAGTAC
```

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

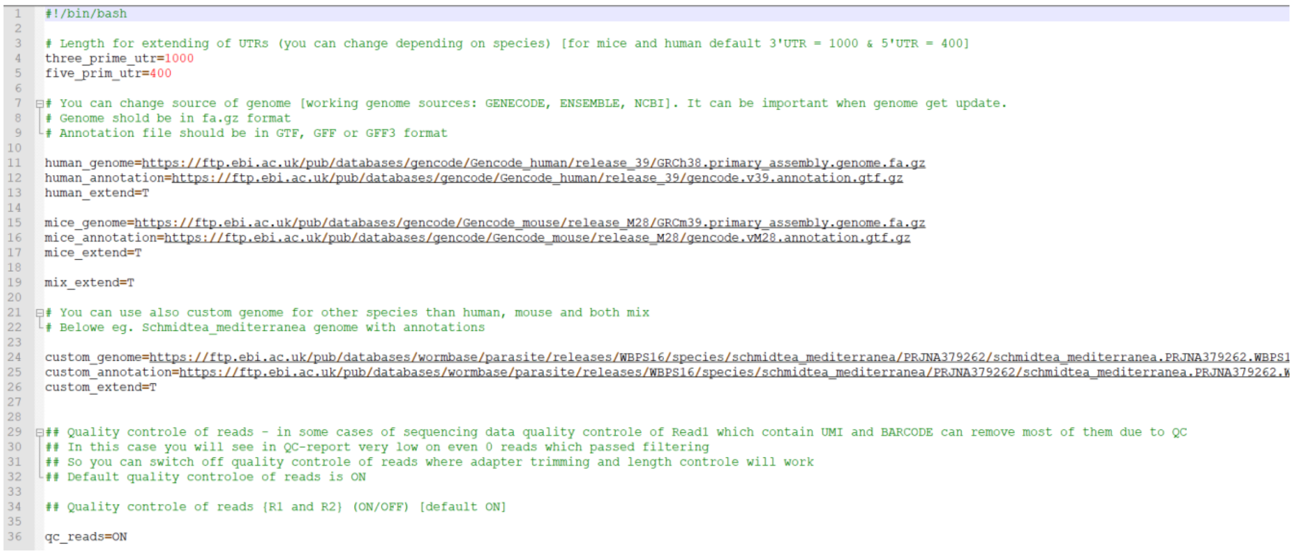
Configuration files:

In the file config_file.conf are included additional parameters necessary during analysis.

```
# Tresholds
## Percent of mitochondiral genes per cell [default 25%]

mt_per:25

## Down threshold for genes per cell [default 500 genes/cell]

down:100

## Up threshold for genes per cell [default NA]

up:6000

#Subtypes selection [CSSG]
## Mitochondrial genes in subtype creation (exclude, include) [default Exclude]

mt_cssg:exclude

## Markers p_val [default 0.05]

m_val:0.05

## Maximum amount of input genes for cluster [default 5000]

max_genes:5000

## Maximum start combination for iteration [default 100]

max_combine:100

## Value for nonclassified cells in cluster

loss_pval:0.001

## Split factor  (20-100) [default 75]

s_factor:75

## Cell contetnt binary test p_value [default 0.1 - in case of rare subpopulations]

p_bin:0.1
```

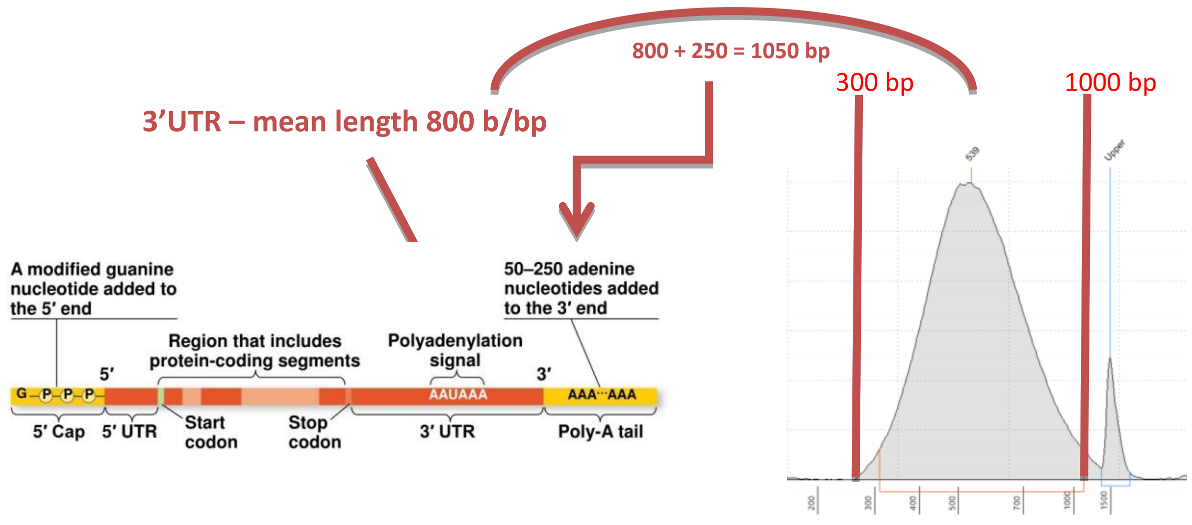
*config_file.conf was added in JSEQ® v.2.1.1 to allow users to control the threshold setting and CSSG cell subtypes creation depending on the type of analysis.

In file genome.conf are included additional parameters necessary for the type of genome choosing and improvement of the mapping process.



```
#!/bin/bash

# Length for extending of UTRs (you can change depending on species) [for mice and human default 3'UTR = 1000 & 5'UTR = 400]
three_prime_utr=1000
five_prim_utr=400

# You can change source of genome [working genome sources: GENECODE, ENSEMBLE, NCBI]. It can be important when genome get update.
# Genome shold be in fa.gz format
# Annotation file should be in GTF, GFF or GFF3 format

human_genome=https://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_39/GRCh38.primary_assembly.genome.fa.gz
human_annotation=https://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_39/gencode.v39.annotation.gtf.gz
human_extend=T

mice_genome=https://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_mouse/release_M28/GRCm39.primary_assembly.genome.fa.gz
mice_annotation=https://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_mouse/release_M28/gencode.vM28.annotation.gtf.gz
mice_extend=T

mix_extend=T

# You can use also custom genome for other species than human, mouse and both mix
# Belowe eg. Schmidtea_mediterranea genome with annotations

custom_genome=https://ftp.ebi.ac.uk/pub/databases/wormbase/parasite/releases/WBPS16/species/schmidtea_mediterranea/PRJNA379262/schmidtea_mediterranea.PRJNA379262.WBPS1
custom_annotation=https://ftp.ebi.ac.uk/pub/databases/wormbase/parasite/releases/WBPS16/species/schmidtea_mediterranea/PRJNA379262/schmidtea_mediterranea.PRJNA379262.W
custom_extend=T


## Quality controle of reads - in some cases of sequencing data quality controle of Read1 which contain UMI and BARCODE can remove most of them due to QC
## In this case you will see in QC-report very low on even 0 reads which passed filtering
## So you can switch off quality controle of reads where adapter trimming and length controle will work
## Default quality controloe of reads is ON

## Quality controle of reads {R1 and R2} (ON/OFF) [default ON]

qc_reads=ON
```

Additionally, JSEQ 2.3.1 was added as an option for annotation file enrichment. It is connected to the single-cell library structure. In most cases, the single-cell library is created from 3'UTR side, where firstly, we have the polyA tail, the next 3'UTR sequence, and then the rest of the transcript. Annotation files do not always provide information about the 3'UTR sequence, so we prepared scripts for extending UTRs, which improved the mapping process to genes. A range of library sequences length causes a lot of sequences to contain UTRs sequences, and when we have no UTR information in the annotation file, the read will be mapped in the intergenic region, and we lose information about the additional count. So, right now, you can choose if you want to extend UTRs sequences, and when yes, you can choose the length to extend for both (if the length is possible to add).


800 + 250 = 1050 bp
300 bp
1000 bp
3'UTR – mean length 800 b/bp
A modified guanine nucleotide added to the 5' end
50–250 adenine nucleotides added to the 3' end
Region that includes protein-coding segments
Polyadenylation signal
5'
3'
G–P–P–P
AAUAAA
AAA···AAA
5' Cap
5' UTR
Start codon
Stop codon
3' UTR
Poly-A tail
539
Upper
200
300
400
500
700
1000
1500

Markers:

Go to file markers_*.xlsx and check markers.

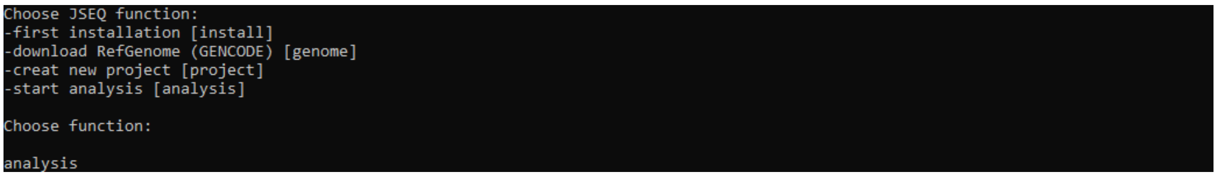
We prepared two sets of markers: markers_brain.xlsx, which contains markers for the mature brain, and markers_developing_brain.xlsx for developing brain and brain organoids. You can also choose non_canonical.xlsx and change the naming approach without using any canonical markers. You can read how cell naming works with markers in part **6.10. Cells naming – markers & 6.11. Construction of cell names**

Fastq analysis includes:

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

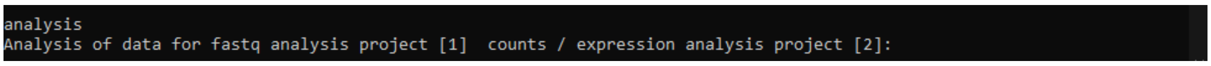
Analysis:

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



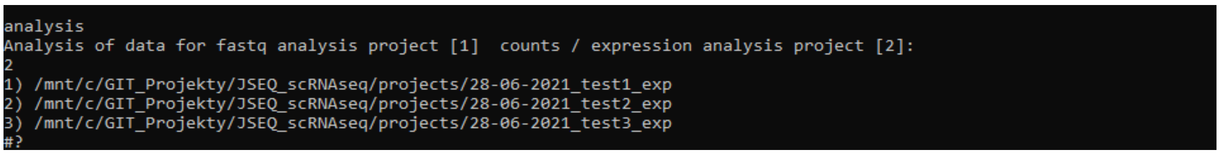
```
Choose JSEQ function:
-first installation [install]
-download RefGenome (GENCODE) [genome]
-creat new project [project]
-start analysis [analysis]

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 analyze. 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 the projects directory 'project/*project_name*/results/process.log.out'. You can check and refresh this log file in real-time during analysis. In the same directory, you can see reads quality control report and a final single-cell analysis report

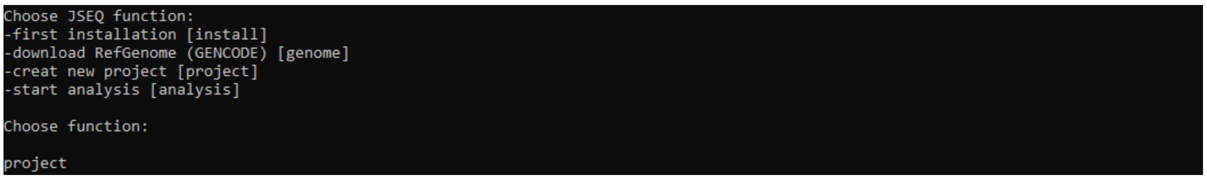
3. Analysis – gene count / normalized expression

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

3.1. Create project

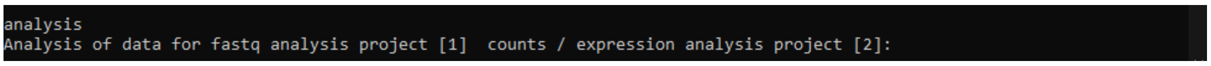
Project creating:

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



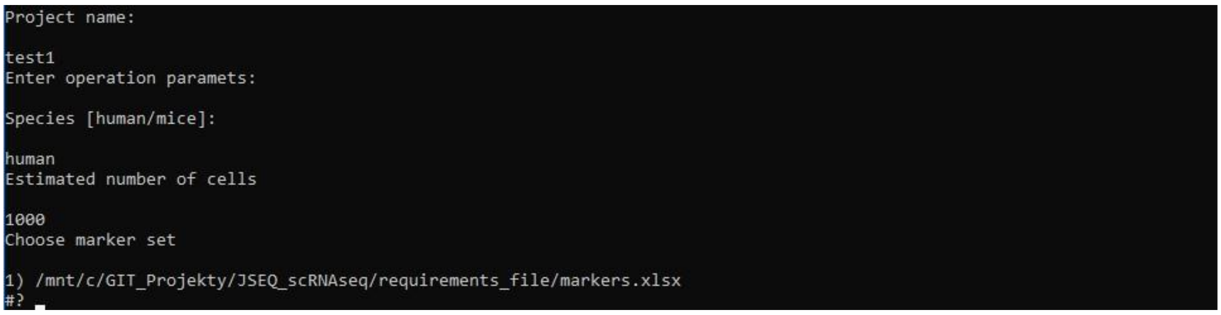
```
analysis
Analysis of data for fastq analysis project [1]  counts / expression analysis project [2]:
```

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 word, then use '_' instead of 'space'.

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

Marker set – choose the right markers set, which will be used in the current 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

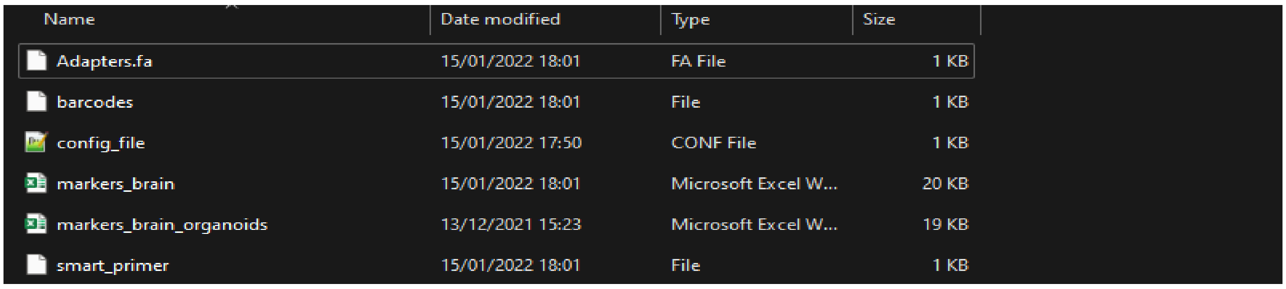
Important: When you use the sparse matrix as input, file names must be for genes - genes.tsv, cell names or barcodes - barcodes.tsv and sparse matrix - matrix.mtx. If not, you will not go to the next step.

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

You can run the first analysis when your project and data are ready.

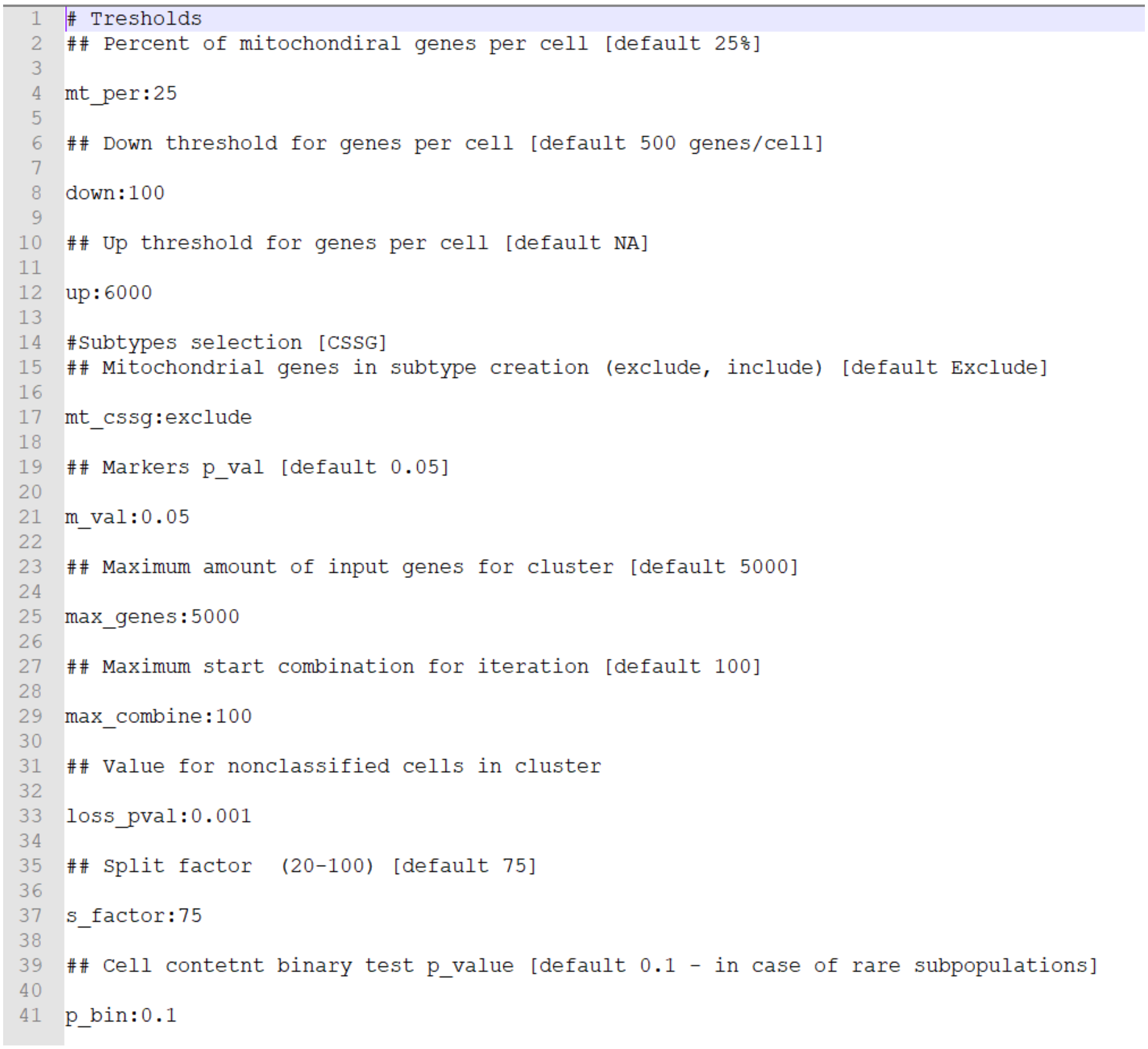
Make sure that the used markers are proper for your analysis. Go to JSEQ_scRNAseq/requirements_file/* and check this.



Name	Date modified	Type	Size
Adapters.fa	15/01/2022 18:01	FA File	1 KB
barcodes	15/01/2022 18:01	File	1 KB
config_file	15/01/2022 17:50	CONF File	1 KB
markers_brain	15/01/2022 18:01	Microsoft Excel W...	20 KB
markers_brain_organoids	13/12/2021 15:23	Microsoft Excel W...	19 KB
smart_primer	15/01/2022 18:01	File	1 KB

Configuration file:

In the file config_file.conf are included additional parameters necessary during analysis.



```
# Tresholds
## Percent of mitochondiral genes per cell [default 25%]

mt_per:25

## Down threshold for genes per cell [default 500 genes/cell]

down:100

## Up threshold for genes per cell [default NA]

up:6000

#Subtypes selection [CSSG]
## Mitochondrial genes in subtype creation (exclude, include) [default Exclude]

mt_cssg:exclude

## Markers p_val [default 0.05]

m_val:0.05

## Maximum amount of input genes for cluster [default 5000]

max_genes:5000

## Maximum start combination for iteration [default 100]

max_combine:100

## Value for nonclassified cells in cluster

loss_pval:0.001

## Split factor  (20-100) [default 75]

s_factor:75

## Cell contetnt binary test p_value [default 0.1 - in case of rare subpopulations]

p_bin:0.1
```

*config_file.conf was added in JSEQ® v.2.1.1 to allow users to control the threshold setting and CSSG cell subtypes creation depending on the type of analysis.

Markers:

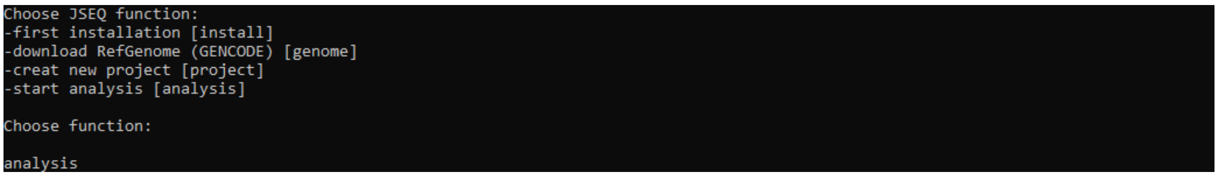
Go to file markers_*.xlsx and check markers. How works cell naming with markers, you can read in part **6.10. Cells naming – markers & 6.11. Construction of cell names**

Counts /expression analysis include:

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

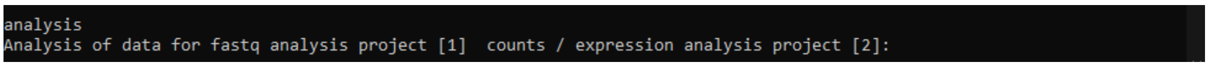
Analysis:

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



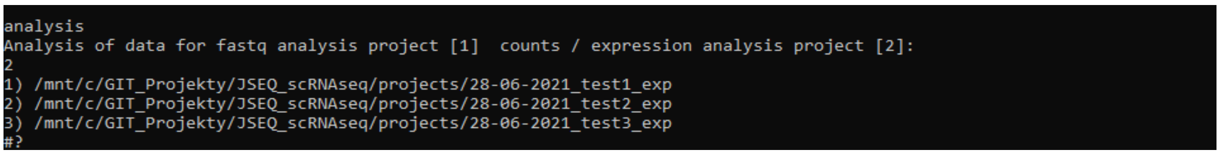
```
Choose JSEQ function:
-first installation [install]
-download RefGenome (GENCODE) [genome]
-creat new project [project]
-start analysis [analysis]

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 analyze. 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
#?
```

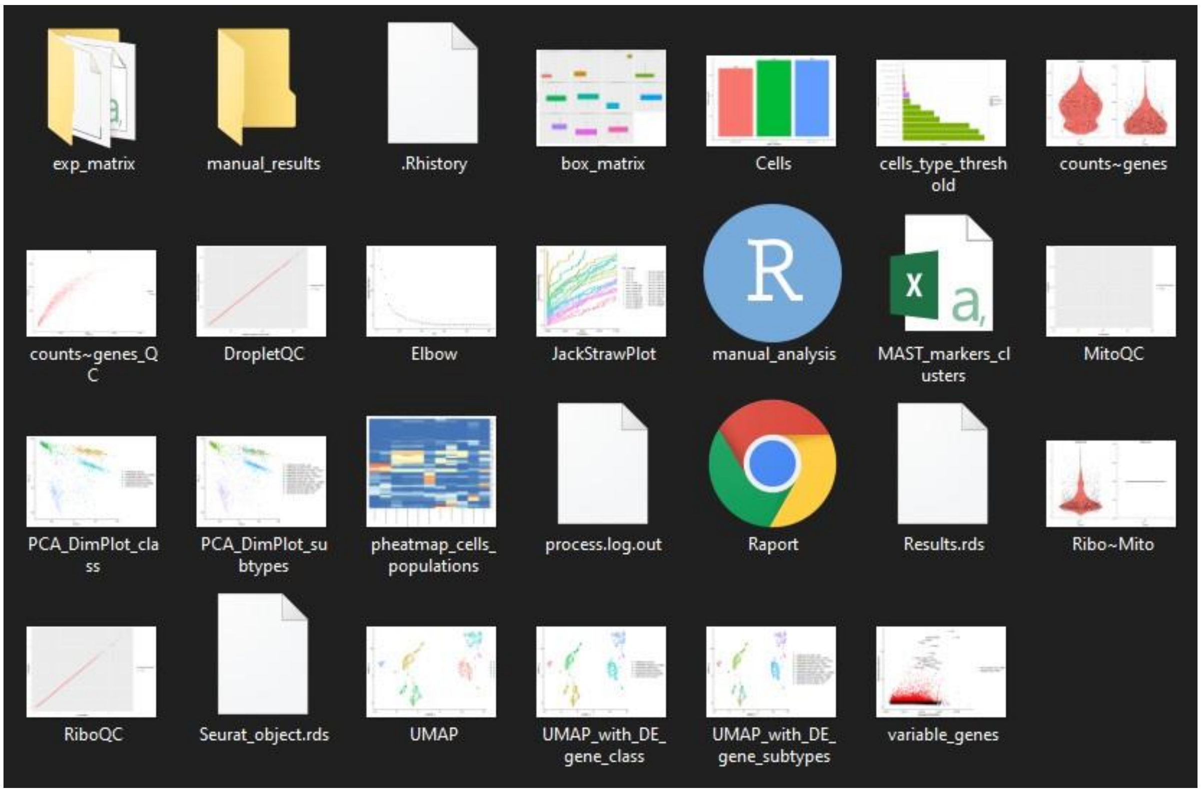
It can last a while; some information you can see in the console, but if you want to check that all works correctly, go to the projects directory 'project/*project_name*/results/process.log.out'. You can check and refresh this log file in real-time during analysis. In the same directory, you can see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

4. Manual analysis

In some cases, automatically obtained results could be inefficient (e.g., wrong cell names, wrong number of principal components (PC), etc.). In such a case, you can run a manual analysis. If the pipeline has finished work, go to the project directory and run manual analysis in RStudio (recommended) or other R language IDE.

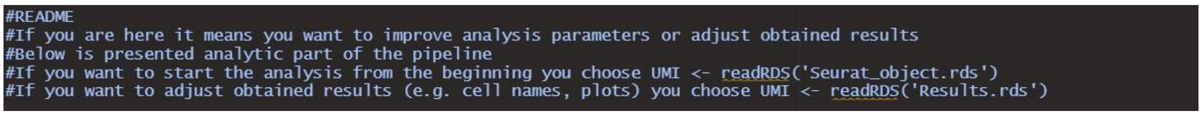
4.1. Run manual analysis

4.1.1. Go to 'project/*project_name*/results/'



4.1.2. Run 'manual_analysis.R'

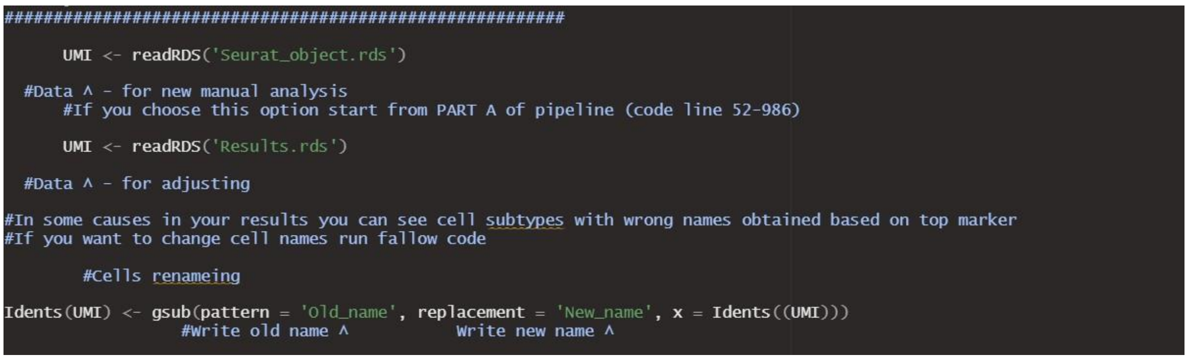
4.1.3. Read information inside of the script and fill required data



```
#README
#If you are here it means you want to improve analysis parameters or adjust obtained results
#Below is presented analytic part of the pipeline
#If you want to start the analysis from the beginning you choose UMI <- readRDS('Seurat_object.rds')
#If you want to adjust obtained results (e.g. cell names, plots) you choose UMI <- readRDS('Results.rds')
```

4.1.4. Start manual analysis

You can choose different analysis steps from the beginning or only adjust obtained results such as cell names – all information in script '#' tips.



```
#########################################################

      UMI <- readRDS('Seurat_object.rds')

  #Data ^ - for new manual analysis
      #If you choose this option start from PART A of pipeline (code line 52-986)

      UMI <- readRDS('Results.rds')

  #Data ^ - for adjusting

#In some causes in your results you can see cell subtypes with wrong names obtained based on top marker
#If you want to change cell names run fallow code

        #Cells renameing

Idents(UMI) <- gsub(pattern = 'Old_name', replacement = 'New_name', x = Idents((UMI)))
                  #Write old name ^            Write new name ^
```

5. Report & Analysis

5.1. Quality control of the reads

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

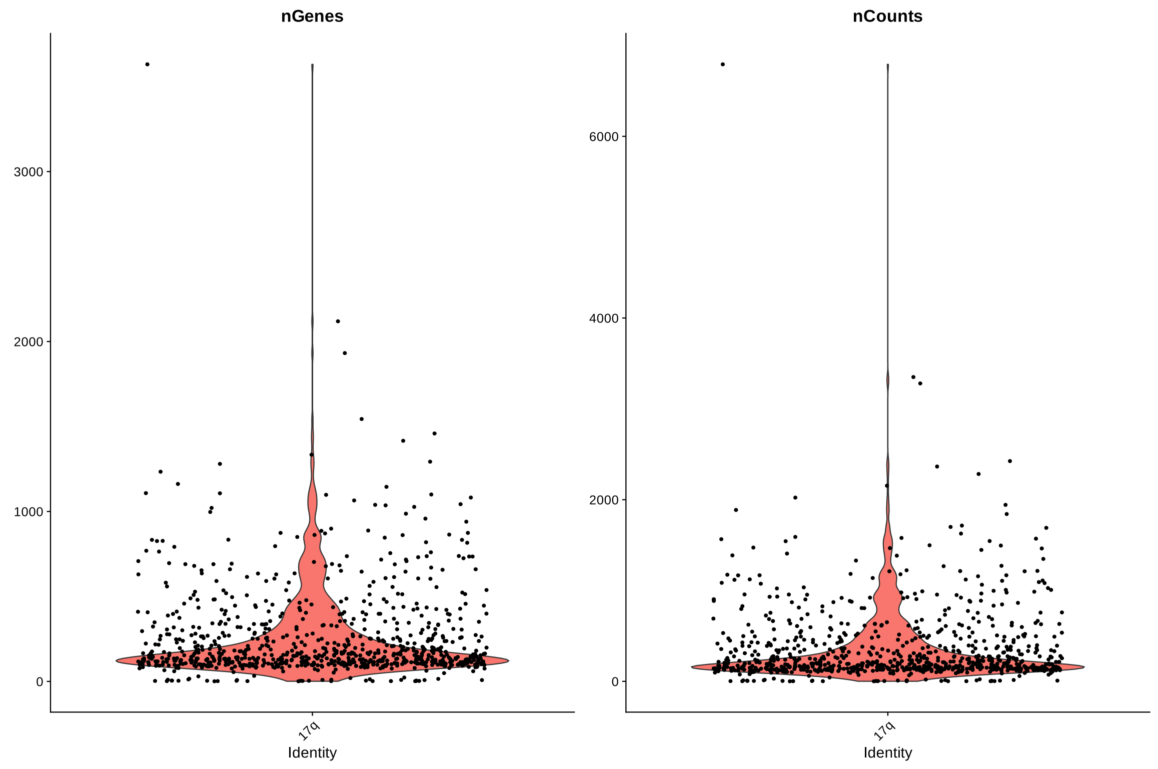
- Adapters content
- Adapters sequence
- Bad ligation adapters
- Nucleotide content graphs

6. Single-cell analysis & statistics

At this stage, many analyses are based on the Seurat library - StjaliLab library with elements that I have programmed 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 cell clusters. More information will be provided later.

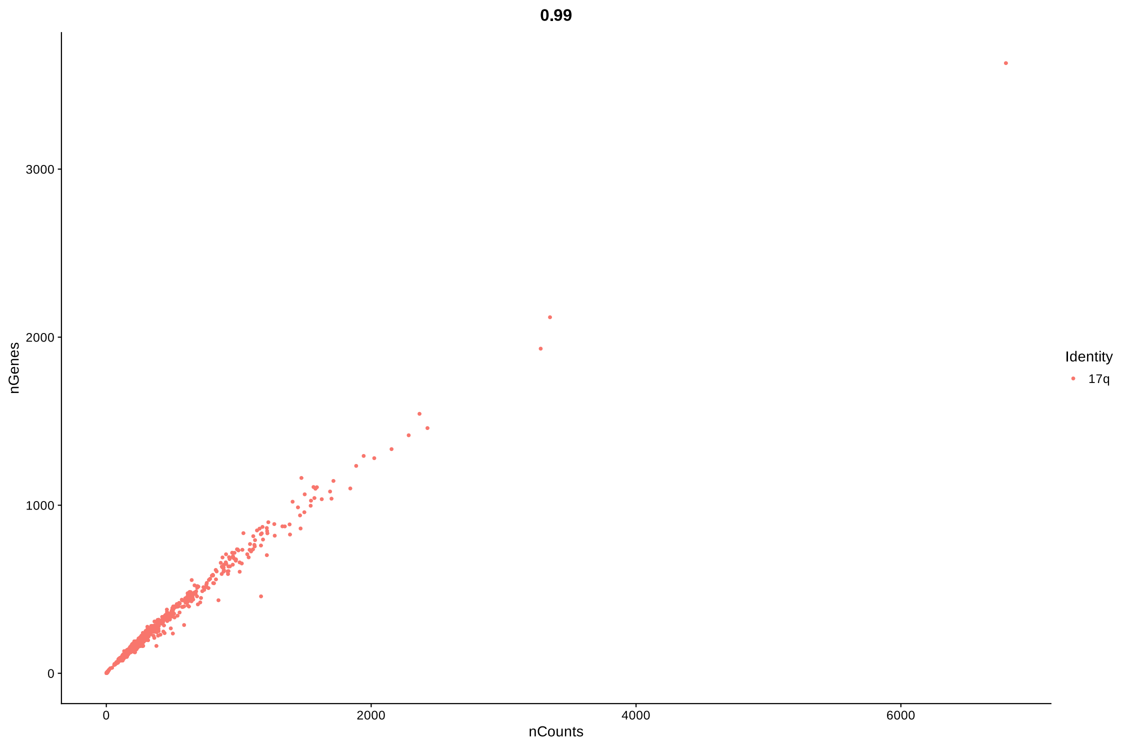
6.1. Cells content information

Counts ~ genes - density plot:



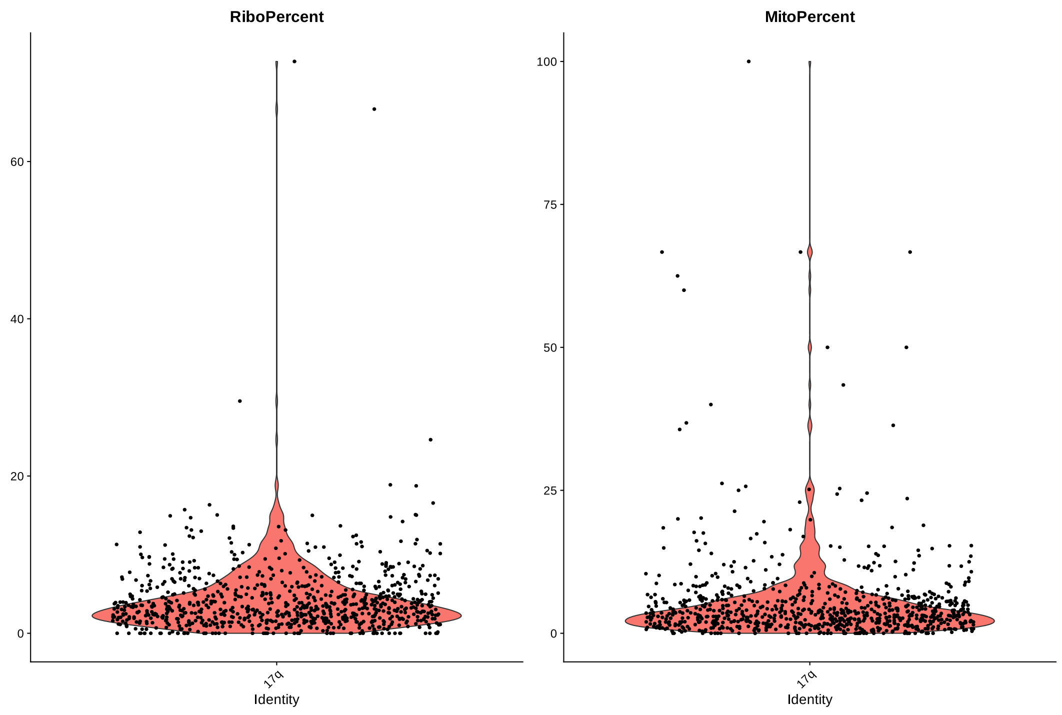
These plots show the density for the number of counts and identified genes for each cell in the analysis.

Counts ~ genes - correlation plot:



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

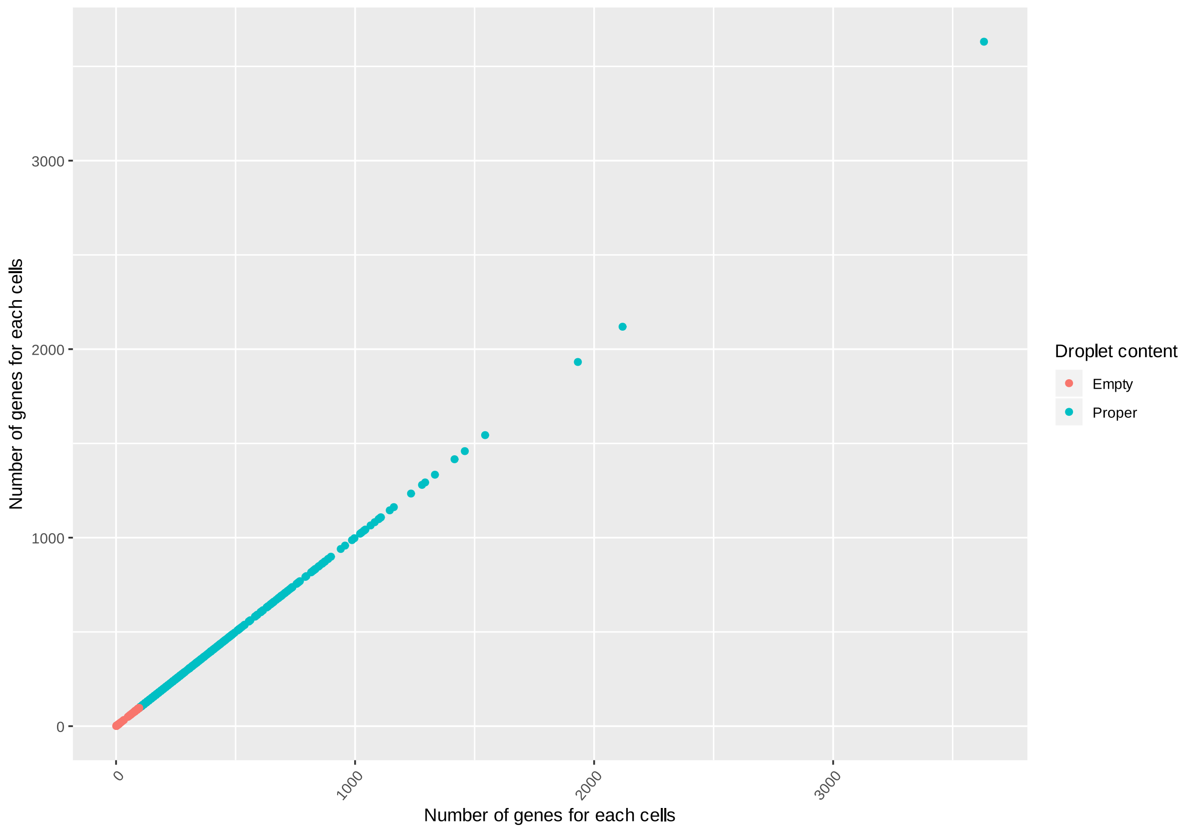
Percent of mitochondrial and ribosomal genes – density plot:



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

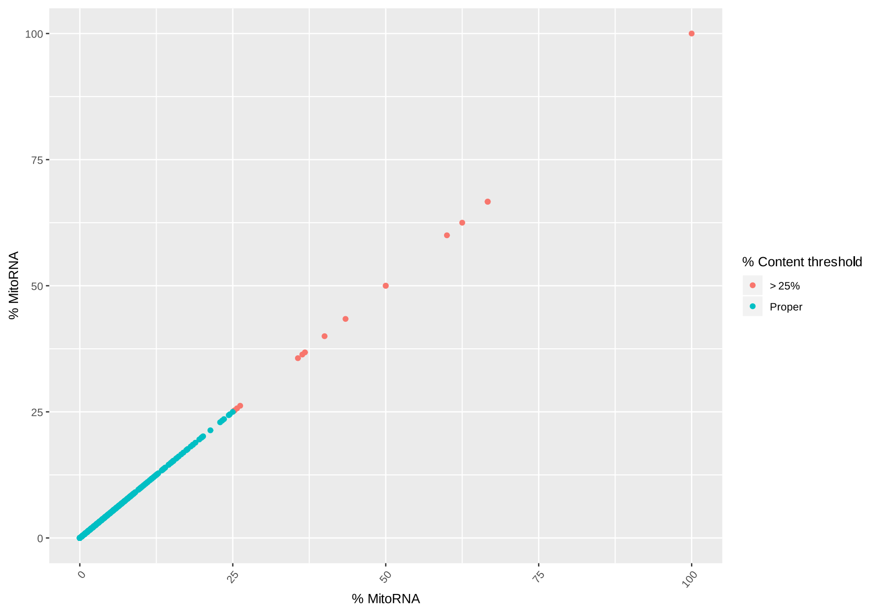
6.2. Outliers and thresholds

Genes number plot:



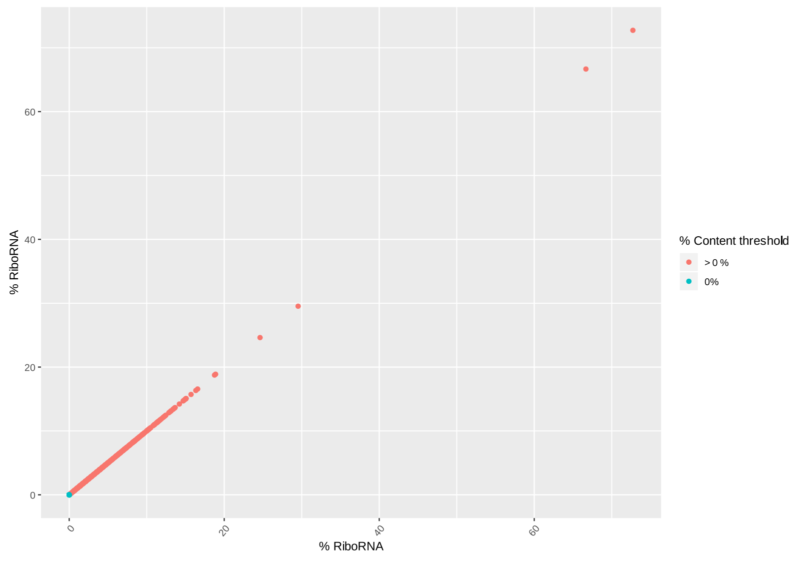
This plot shows compare the number of genes to the number of genes for each cell. Thanks to this, we can see outliers' values. Default the threshold is set for bottom values on a minimum of 500 genes per cell, and for maximal values is calculated based on two times mean value plus one and half times the interquartile range (2*MEAN + 1,5*IQR) as the maximum value, when in the config_file.conf variable UP:NA is setting. Cells below bottom values are recognized as empty cells; upper top values are doublets, triplets, etc.

Mitochondrial genes content – plot:



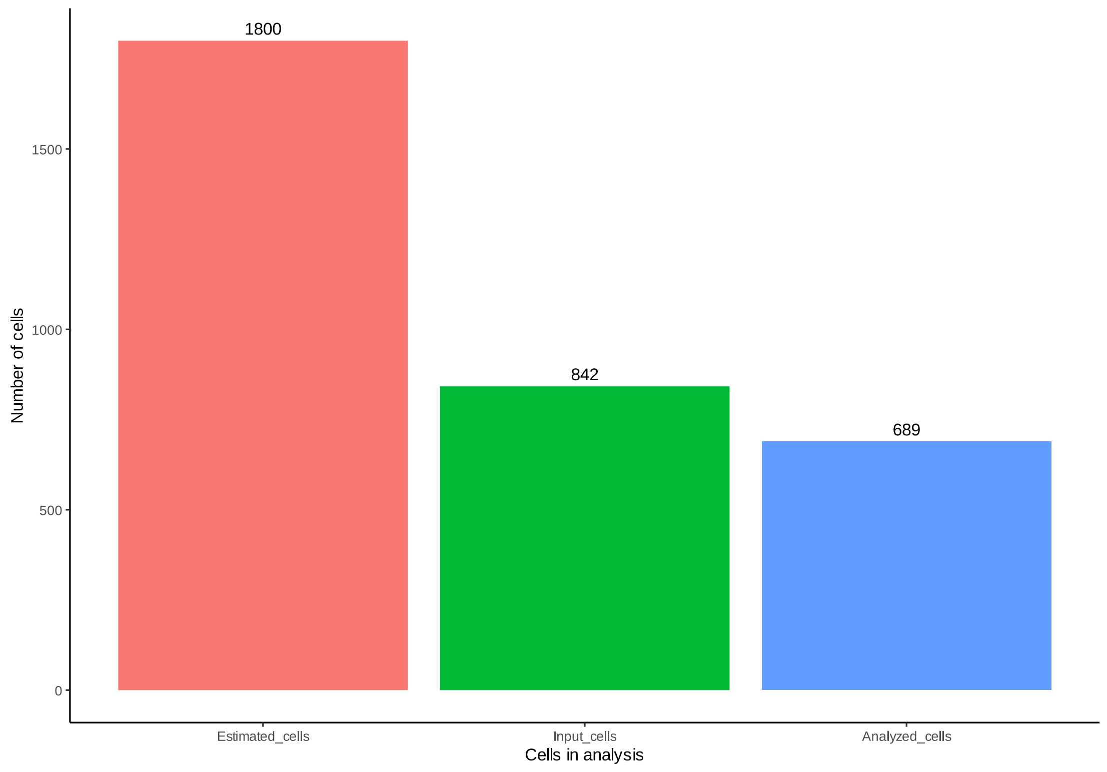
The mitochondrial gene content threshold default is set at 25% of all genes displayed in a single cell. All cells that display mitochondrial genes greater than 25% are removed.

Ribosomal genes content – plot:



For ribosomal genes, there are no set thresholds. This plot shows only ribosomal gene content equal to 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 the estimated amount of cells and cells amount at the beginning of analysis and after quality control with set thresholds.

6.3. Data normalization

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

Formula:

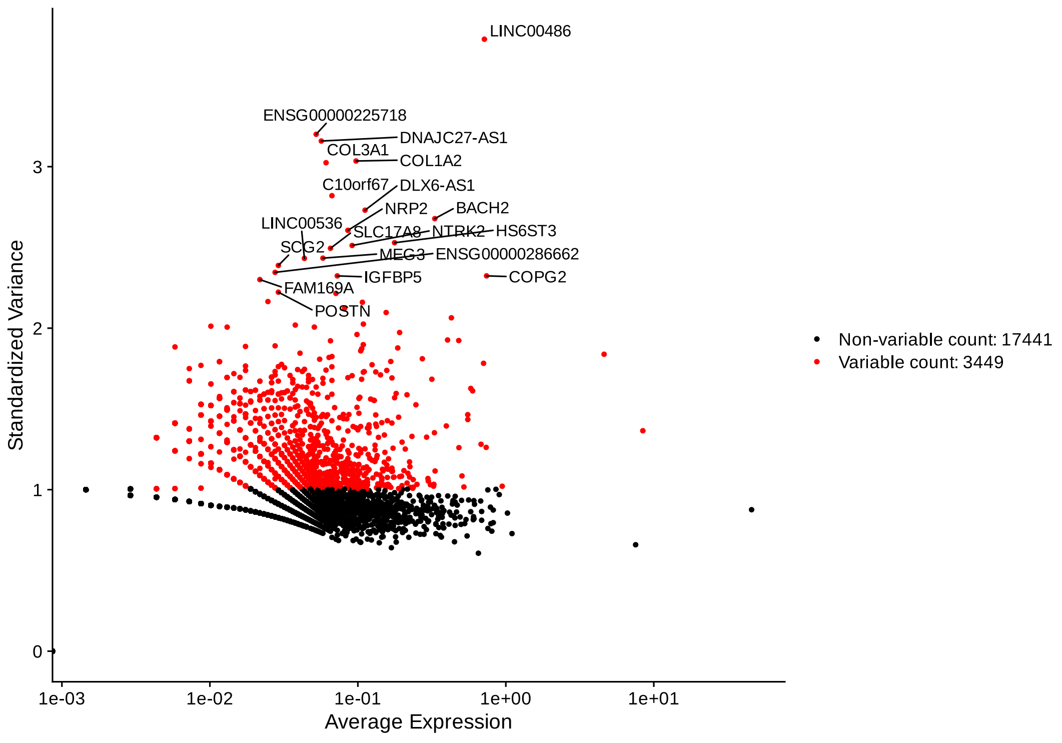
$$CPM = \frac{\frac{\text{count of genes}}{\text{sum of counts per cell}}}{1000000}$$

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

6.4. Most variable genes

To calculate the most variable genes, the 'vst' (Variance Stabilizing Transformation) selection method was used with the 'equal_frequency' method based on the Seurat function FindVariableFeatures.

Most variable genes – plot:



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

6.5. Principle components (PCs)

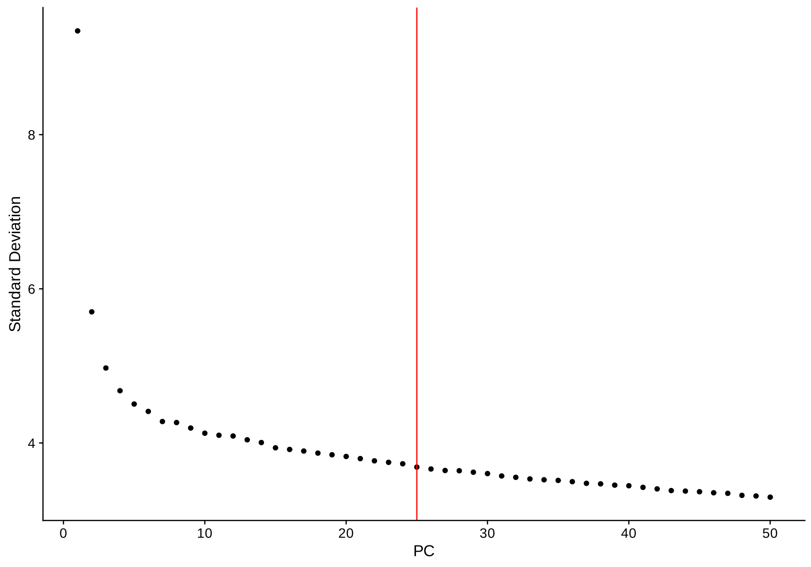
Principle components are a method that is responsible for reducing the dimensionality of 'p' numerical variables for each 'n' element, increasing interpretability without losing any information (Jolliffe & Cadima, 2016). Thanks to this method, we can deal with gene expression matrix for a great number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

Principal components were calculated on scaled data (ScaleData function) with most variable features (genes) using the Seurat function 'RunPCA'.

6.6. Selecting the right number of PCs

The selection was based on the Elbow plot, JackStraw plot, and a special algorithm that checked changes in standard deviation between the following PCs to select the right number of principal components in the analysis. The Elbow plot was created using Seurat functions' ElbowPlot' with the maximal number of dimension 50.

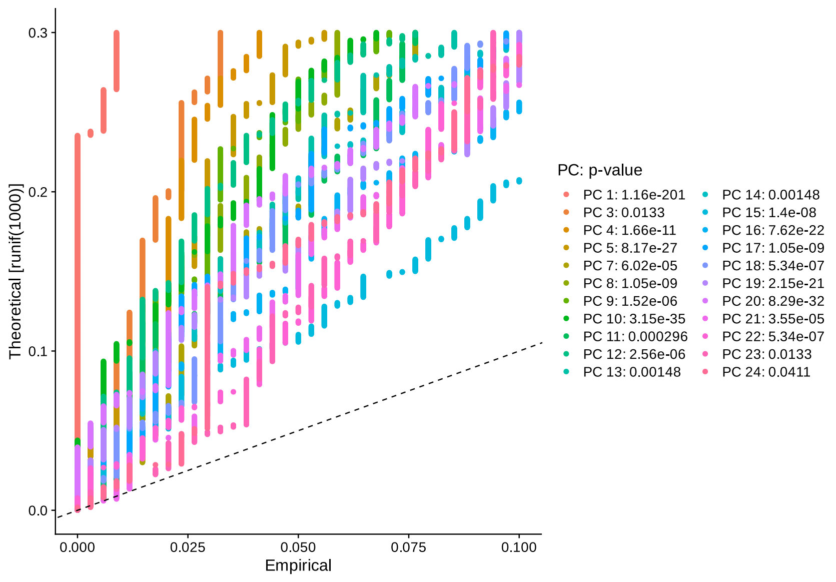
ElbowPlot:



In this case, the algorithm chose 25 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 PC (p_val = 0.05).

JackStraw plot:



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

6.7. Data clustering

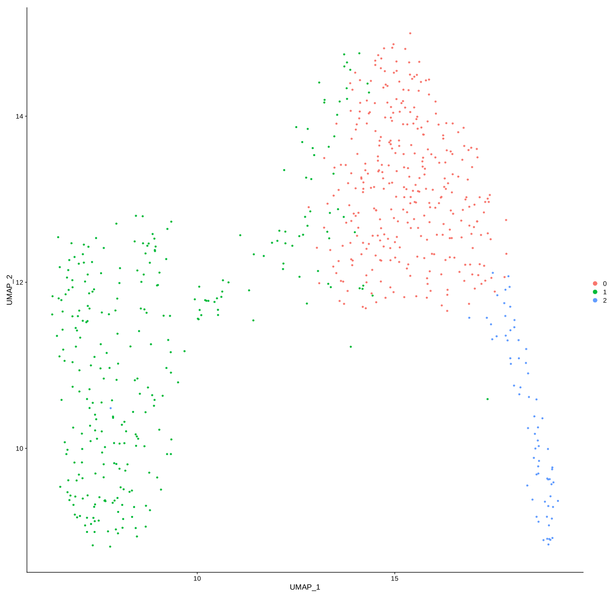
Data clustering based on previously selected PCs uses two Seurat functions: 'FindNeighbors' and 'FindClusters'. These functions are based on KNN (k-nearest neighbor) and SNN (shared nearest neighbor) algorithms. FindCluster function is set on resolution 0.5, the number of starts 10, and the number of iterations 1000.

Both algorithms are common for single-cell analysis and provide clusters connected with different cell populations (Zhu et al., 2020). In the following 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.

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

In contrast to traditional linear dimensionality reduction methods like PCA is non-linear UMAP. The UMAP method is another method based on dimensionality reduction and is similar to tSNE method and also belongs to the non-linear visualization method. The main role of UMAP algorithms was single-cell data visualization (Narayan et al., 2020). Furthermore, UMAP seems more convenient than the tSNE method, which can be problematic with large data sets. UMAP optimizes 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.

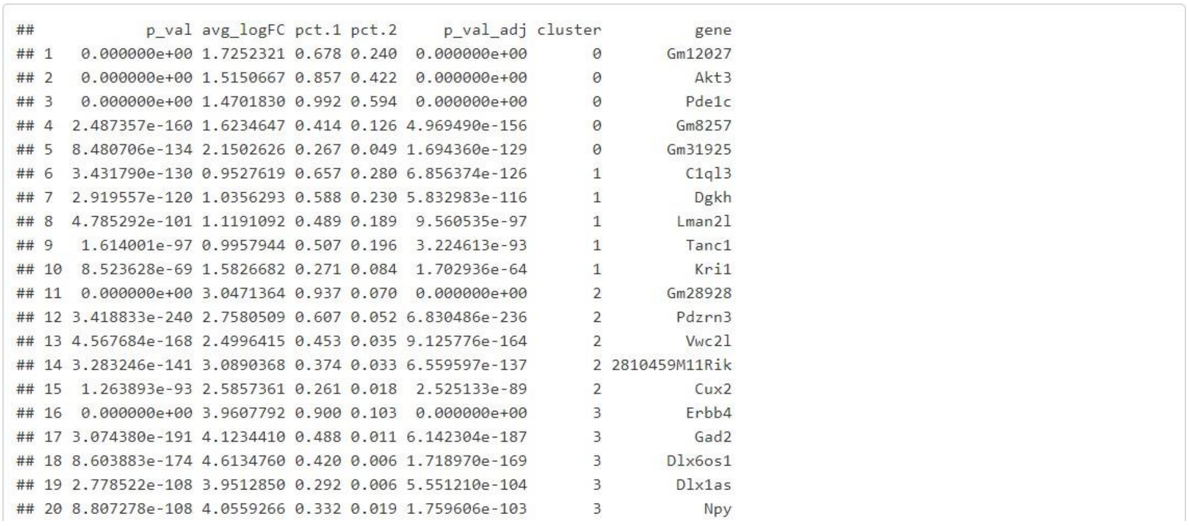
UMAP plot:



6.9. Cluster markers

To obtain specific markers for clusters were used Seurat function 'FindAllMarkers'. The selecting method used the algorithm 'MAST' with only positive markers. MAST algorithm for single-cell gene expression is based on two-part generalized linear models (GLM). The 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
```

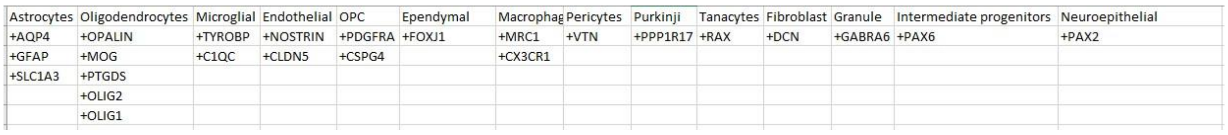
Moreover, these markers will use for dividing subclass clusters into cell subtypes based on the Cell Subtypes Selection by Genes (CSSG) algorithm.

6.10. Cells naming - markers

A particular Cell Clusters Naming (CCN) algorithm was written for cell naming, which checked the most expressed marker for each cluster. There are two approaches for cluster naming. The first approach is based on known, canonical, defined user markers.

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

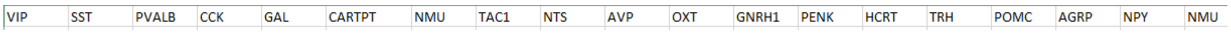
Cell classes markers



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 your 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 be readable. It is an excellent 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

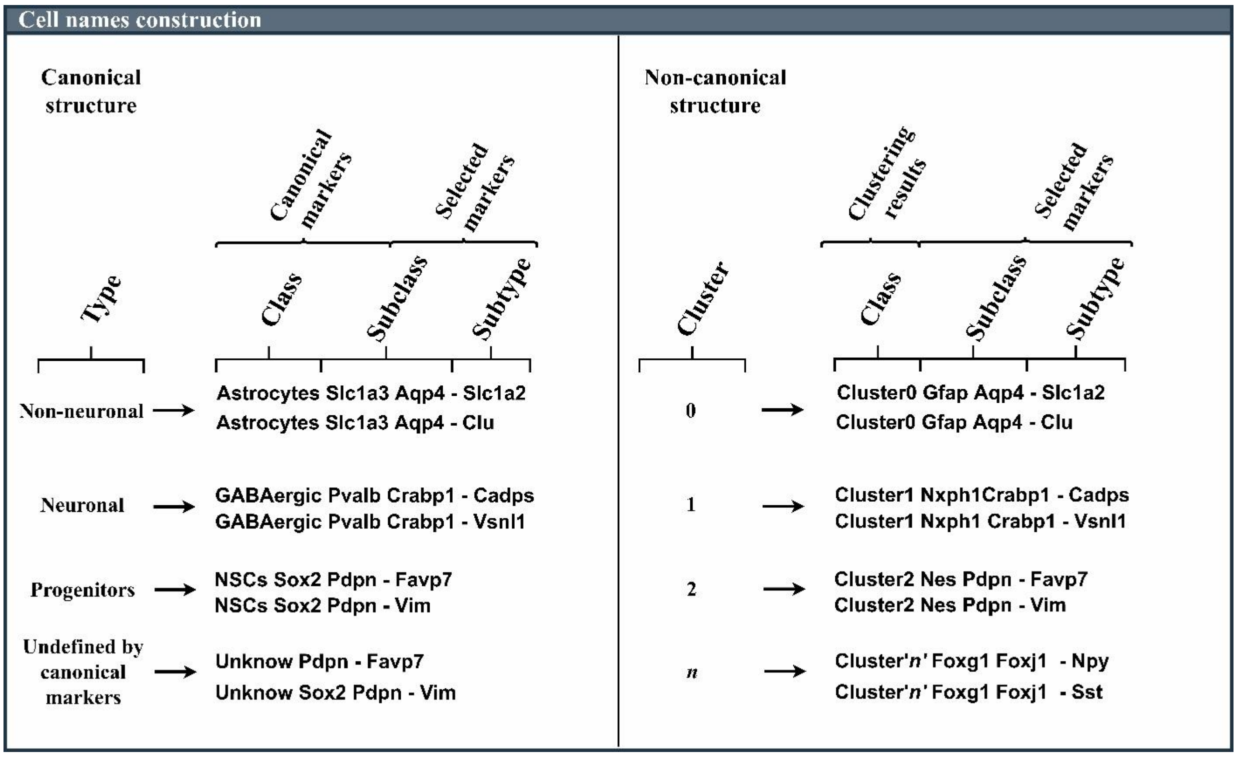
In this case, you need not use additional '+' for markers.

The presented set of markers includes markers that determine non-neuronal and neuronal cell class and additionally markers for neurotransmitters, neuropeptides, their receptors, and calcium-binding proteins as genes involved in interneuronal signaling. Both sets of markers are based on a significant 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 gives the name to the class. In the following steps, the algorithm gives names for cell subclass and subtypes in the same way.

6.11. Construction of cell names

In JSEQ 3.2.1, we have two types of cell naming: for the user who defined a canonical set of markers for cell population naming – canonical name structure, and for the user who wants only to check the structure of the single-cell data without knowledge about cell populations lineage affiliation and attached to the cell names only automatical selected gene markers– non-canonical name structure.

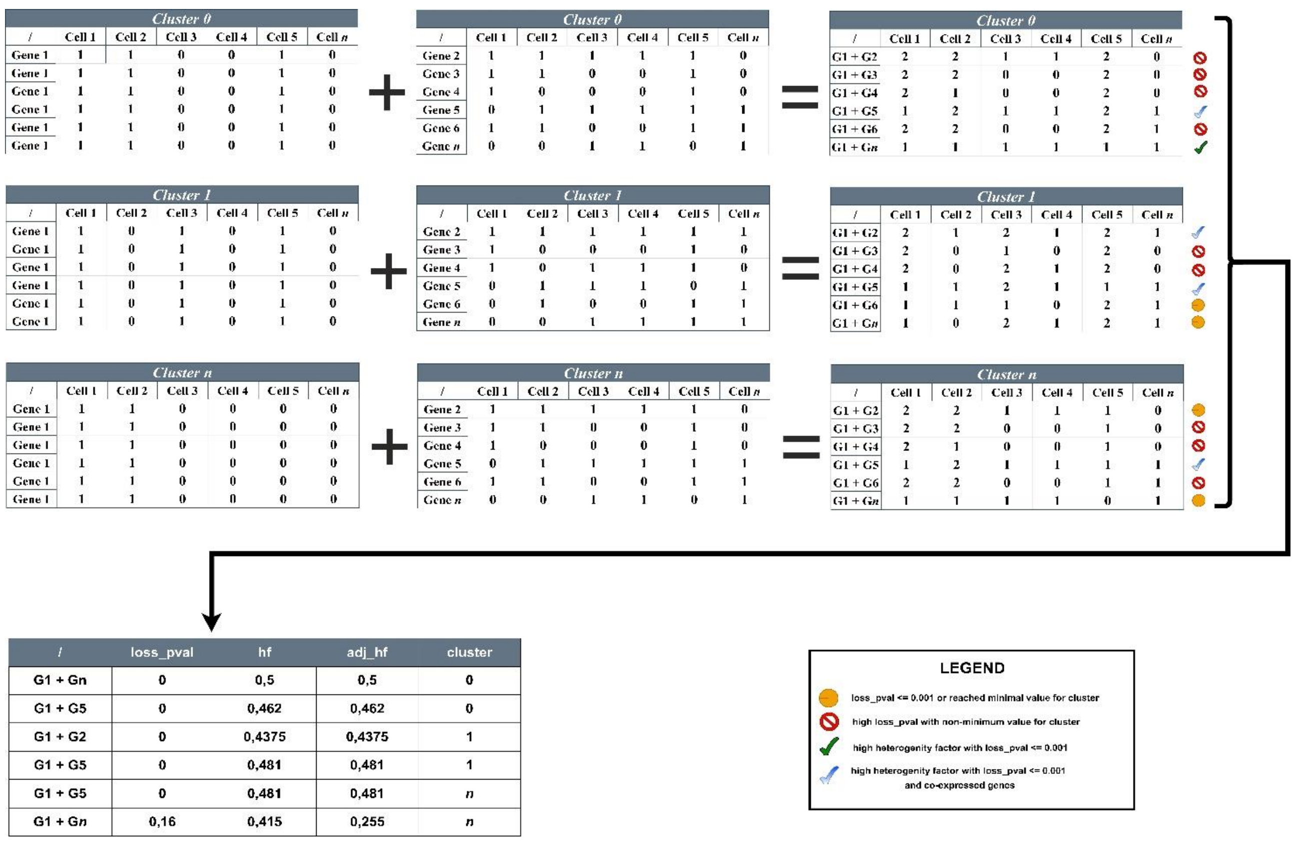


6.12. Dividing the cell subclasses (clusters) into cell subtypes

Due to the high heterogeneity inside clusters obtained in the fundamental single-cell analysis, the Cell Subtypes Selection by Genes (CSSG) algorithm was written. The CSSG algorithm is a very powerful tool based on the diversity of individual gene occurrence inside estimated cell clusters and divides them into smaller, less heterogeneous subsets. The basic regulatory unit of the cell is a gene and its products (protein, splicing forms, non-coding RNAs), so even one gene can have a huge impact on the cell's fate by regulating canonical signaling pathways at different levels. The algorithm can align combinations of single cluster-specific genes to explain a cluster's wide heterogeneity. In this approach, we exclude information about gene expression and transform it into binary data containing information about gene occurrence, where the value '1' means positive gene and '0' means negative gene when it comes to occurrence in every single cell belonging to one cluster. The algorithm uses previously selected by the MAST algorithm (or in a different way) marker genes of each cluster which allows analyzing gene combinations based on cluster-specific genes on a significant level p <= 0.05 of Wilcoxon-Test, which reduces working time and CPU and RAM usage by the algorithm.

During the course of the algorithm's work are conducted iterative series of matches binary information about specific marker genes occurrence in each cluster, where two types of matrices are created. In each series of matches for successive genes, the first matrix contains a replication of one marker gene vector with dimensions the same as the second matrix containing all marker genes occurrence information minus the information about the currently studied gene. Such prepared matrices are summed to obtain each gene occurrence combination inside the cluster. The possible number of combinations is between 1 and the factorial from a max number of marker genes for the cluster at a significant level *p* <= 0.05 of the Wilcoxon-Test $\left(\lim_{Cn\to\infty} C_n \in 1{:}n!\right)$. During the next steps for each row in the summed matrix are calculated statistics (*loss_pval* [*p* value for unexplained cell losses], *ht* [heterogeneity factor], *adj_ht* [adjusted heterogeneity factor]). If the relevant conditions are not obtained, it means *loss_pval* reached the default level of 0.001, or if the next combination did not reduce *loss_pval* or increased *ht*, the next iteration of the combination would start. Next iterations are based on previous iterations summed matrix results, where for the second iteration, input data statistic *ht* should be equal or higher than the third quantile of all *ht* values [*ht* >= Q75], and for the third and next iterations, statistic *loss_pval* should be equal or less than the first quantile of all *loss_pval* [*loss_pval* <= Q25]. Used thresholds are necessary to reduce the number of results to only important combinations for the next iterations due to a great number of possible gene combinations, which can be created during analysis that influence the longer working time of the algorithm and more CPU and RAM usage. The results matrix from the previous iteration is the second matrix for the current iteration sum of matrices; thus, we obtain a combination of more genes until the conditions for completing the analysis are met. If the conditions are reached, all gene combinations determined in the analysis, where *loss_pval* <= Q25 [quantile], are saved to the data frame along with their statistics. Then the cluster sub-setting is created inside each cluster using the dominant expression [max(log(CPM+1)] of genes from the best genes combination based on the *adj_ht* statistic for a given cluster.

The CSSG algorithm workflow



Cluster 0						
/	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell *n*
Gene 1	1	1	0	0	1	0
Gene 1	1	1	0	0	1	0
Gene 1	1	1	0	0	1	0
Gene 1	1	1	0	0	1	0
Gene 1	1	1	0	0	1	0
Gene 1	1	1	0	0	1	0

+

Cluster 0						
/	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell *n*
Gene 2	1	1	1	1	1	0
Gene 3	1	1	0	0	1	0
Gene 4	1	0	0	0	1	0
Gene 5	0	1	1	1	1	1
Gene 6	1	1	0	0	1	1
Gene *n*	0	0	1	1	0	1

=

Cluster 0							
/	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell *n*	
G1 + G2	2	2	1	1	2	0	🚫
G1 + G3	2	2	0	0	2	0	🚫
G1 + G4	2	1	0	0	2	0	🚫
G1 + G5	1	2	1	1	2	1	✓
G1 + G6	2	2	0	0	2	1	🚫
G1 + G*n*	1	1	1	1	1	1	✔

Cluster 1						
/	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell *n*
Gene 1	1	0	1	0	1	0
Gene 1	1	0	1	0	1	0
Gene 1	1	0	1	0	1	0
Gene 1	1	0	1	0	1	0
Gene 1	1	0	1	0	1	0
Gene 1	1	0	1	0	1	0

+

Cluster 1						
/	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell *n*
Gene 2	1	1	1	1	1	1
Gene 3	1	0	0	0	1	0
Gene 4	1	0	1	1	1	0
Gene 5	0	1	1	1	0	1
Gene 6	0	1	0	0	1	1
Gene *n*	0	0	1	1	1	1

=

Cluster 1							
/	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell *n*	
G1 + G2	2	1	2	1	2	1	✓
G1 + G3	2	0	1	0	2	0	🚫
G1 + G4	2	0	2	1	2	0	🚫
G1 + G5	1	1	2	1	1	1	✓
G1 + G6	1	1	1	0	2	1	●
G1 + G*n*	1	0	2	1	2	1	●

Cluster *n*						
/	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell *n*
Gene 1	1	1	0	0	0	0
Gene 1	1	1	0	0	0	0
Gene 1	1	1	0	0	0	0
Gene 1	1	1	0	0	0	0
Gene 1	1	1	0	0	0	0
Gene 1	1	1	0	0	0	0

+

Cluster *n*						
/	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell *n*
Gene 2	1	1	1	1	1	0
Gene 3	1	1	0	0	1	0
Gene 4	1	0	0	0	1	0
Gene 5	0	1	1	1	1	1
Gene 6	1	1	0	0	1	1
Gene *n*	0	0	1	1	0	1

=

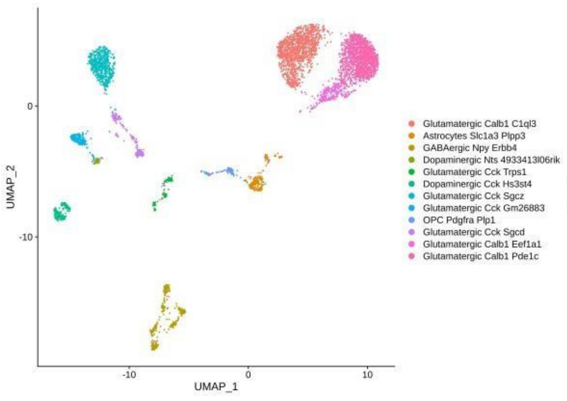
Cluster *n*							
/	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell *n*	
G1 + G2	2	2	1	1	1	0	●
G1 + G3	2	2	0	0	1	0	🚫
G1 + G4	2	1	0	0	1	0	🚫
G1 + G5	1	2	1	1	1	1	✓
G1 + G6	2	2	0	0	1	1	🚫
G1 + G*n*	1	1	1	1	0	1	●

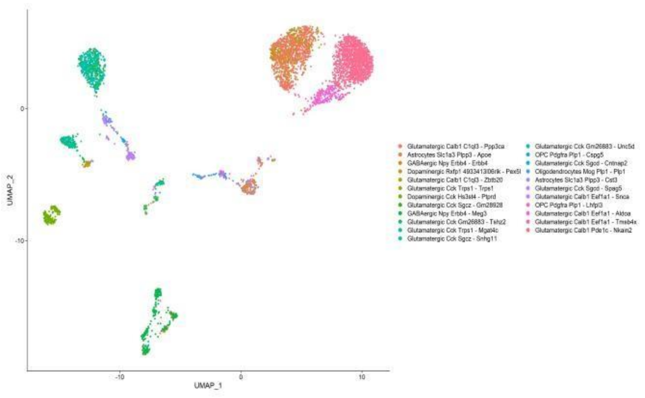
/	loss_pval	hf	adj_hf	cluster
G1 + Gn	0	0,5	0,5	0
G1 + G5	0	0,462	0,462	0
G1 + G2	0	0,4375	0,4375	1
G1 + G5	0	0,481	0,481	1
G1 + G5	0	0,481	0,481	*n*
G1 + G*n*	0,16	0,415	0,255	*n*

LEGEND

●	loss_pval <= 0.001 or reached minimal value for cluster
🚫	high loss_pval with non-minimum value for cluster
✔	high heterogenity factor with loss_pval <= 0.001
✓	high heterogenity factor with loss_pval <= 0.001 and co-expressed genes

Data presentation with and without CSSG algorithm





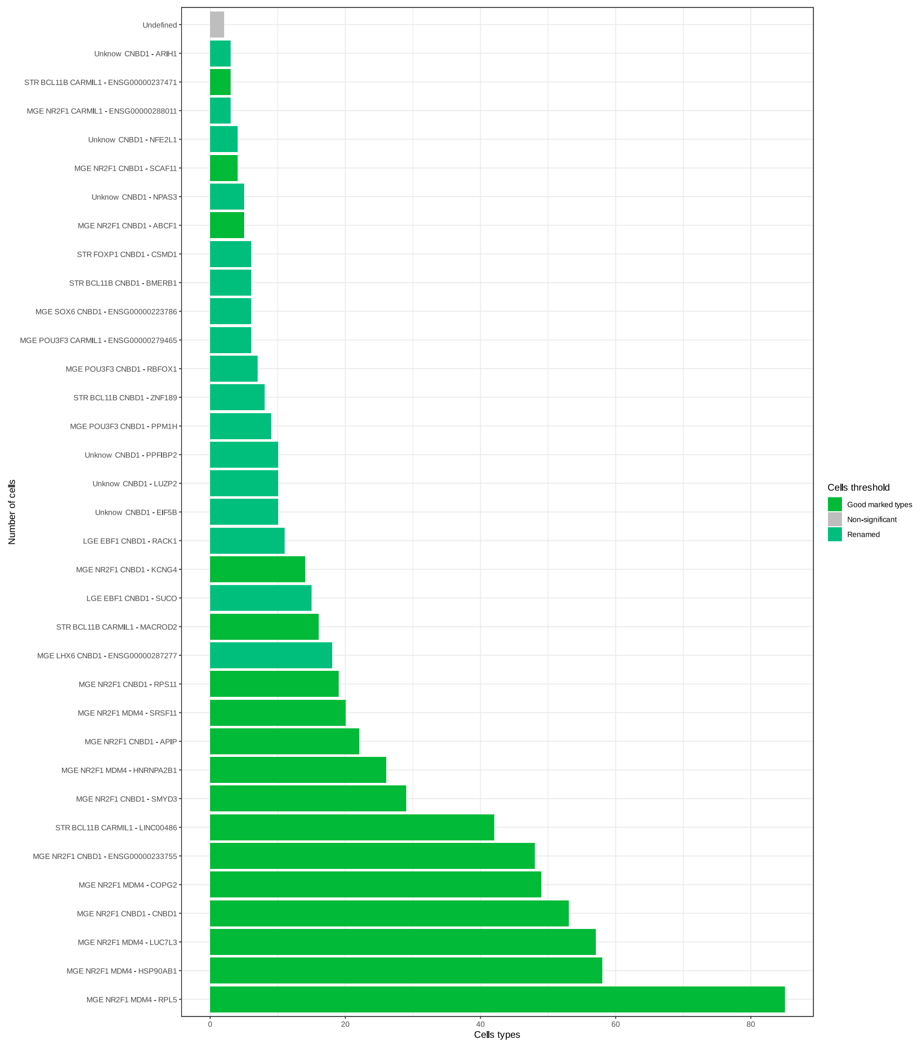
The JSEQ® CSSG algorithm was tested on 812 945 single cells from different brain regions and brain organoids. Results after using the JSEQ® CSSG algorithm show that the algorithm can improve the resolution of obtained results. Based on the obtained result, we can see that algorithm allows:

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

6.13. Outliers dropping

This pipeline contains many checkpoints that protect against lousy quality or badly clustered cells. Even though duplicates removing at the beginning and additional selection points in the pipeline were projected as another quality control step. After dividing cell populations with the CSSG algorithm, obtained cell subtypes in new clusters are checked in terms of proper names. Cell subtypes groups that were poorly marked are renamed to the correct form.
Furthermore, when new cell subtypes do not express markers selected by CSSG, they drop out of the analysis. Moreover, the number of cells on the cell subtypes is controlled by the binomial test. If the number of cells is not statistically significant for a given subtype at a level of 0.1, they are excluded from further analysis. The significance level has been set to 0.1 due to potentially sparse subtypes and may be changed to a lower level in the config file.

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

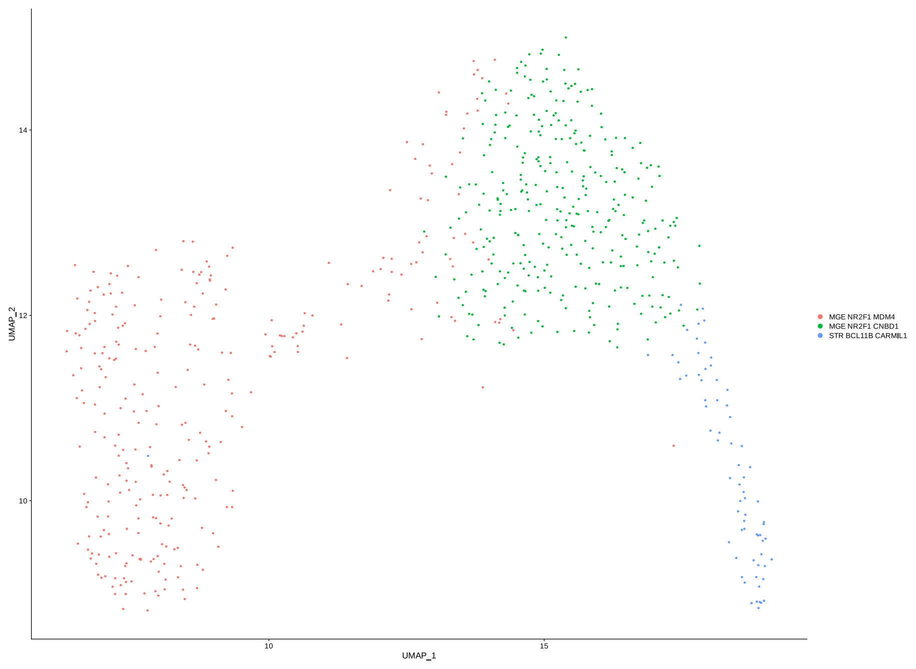


All dropped cells from the analysis are saved to a new dataset in the repository:
'JSEQ_scRNAseq/projects/*project_name*/results/exp_matrix/unknow_cells_count_matrix.xlsx'

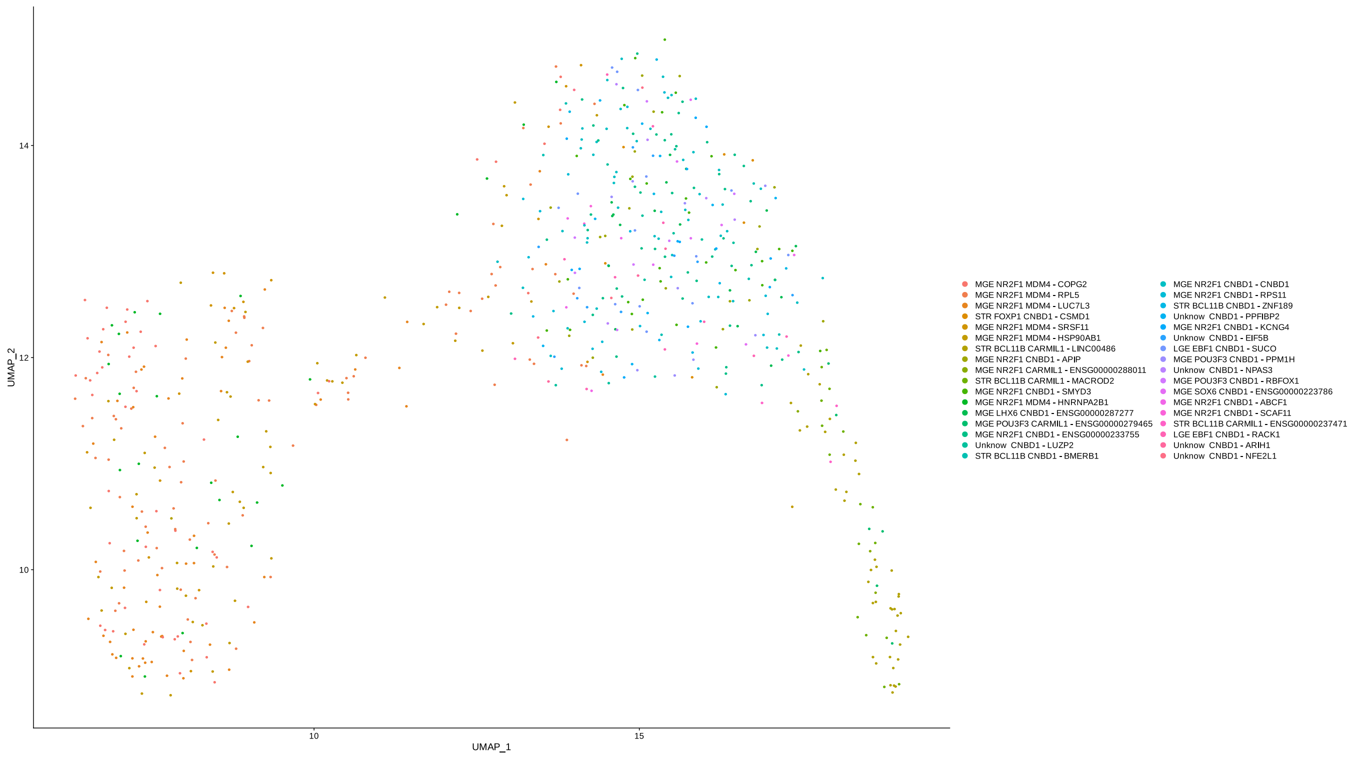
7. Single-cell data visualization

7.1. UMAP – subclass / subtypes

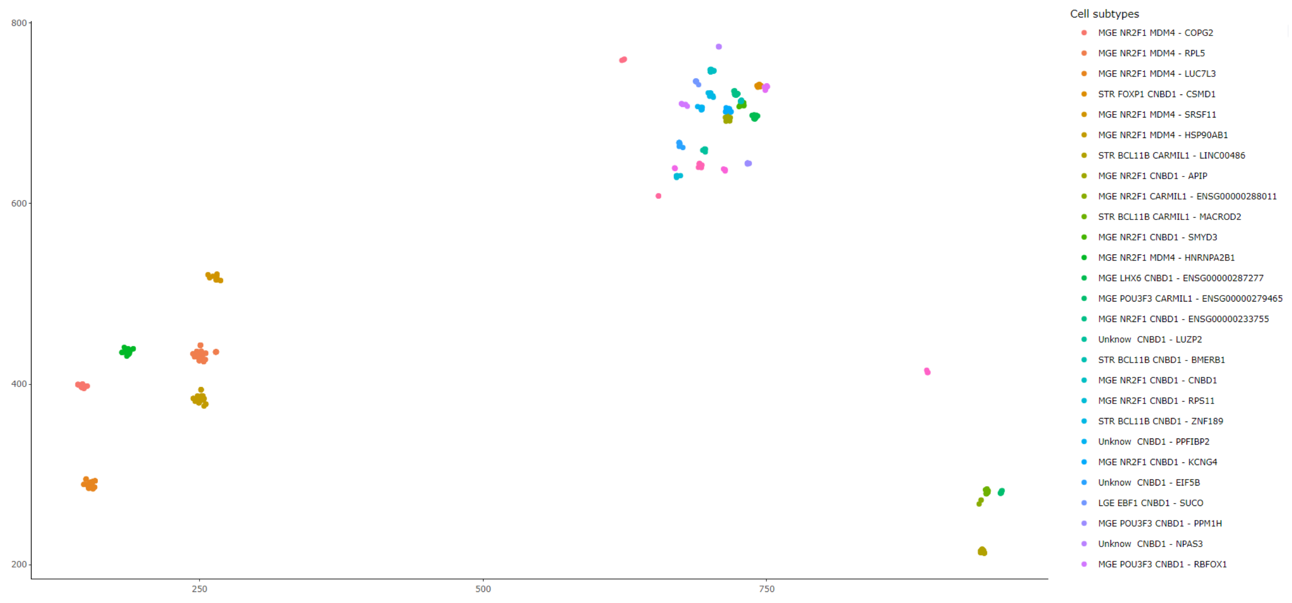
UMAP plot of cell subclasses:



UMAP plot of cell subclasses [subtypes localization on paternal UMAP components]:

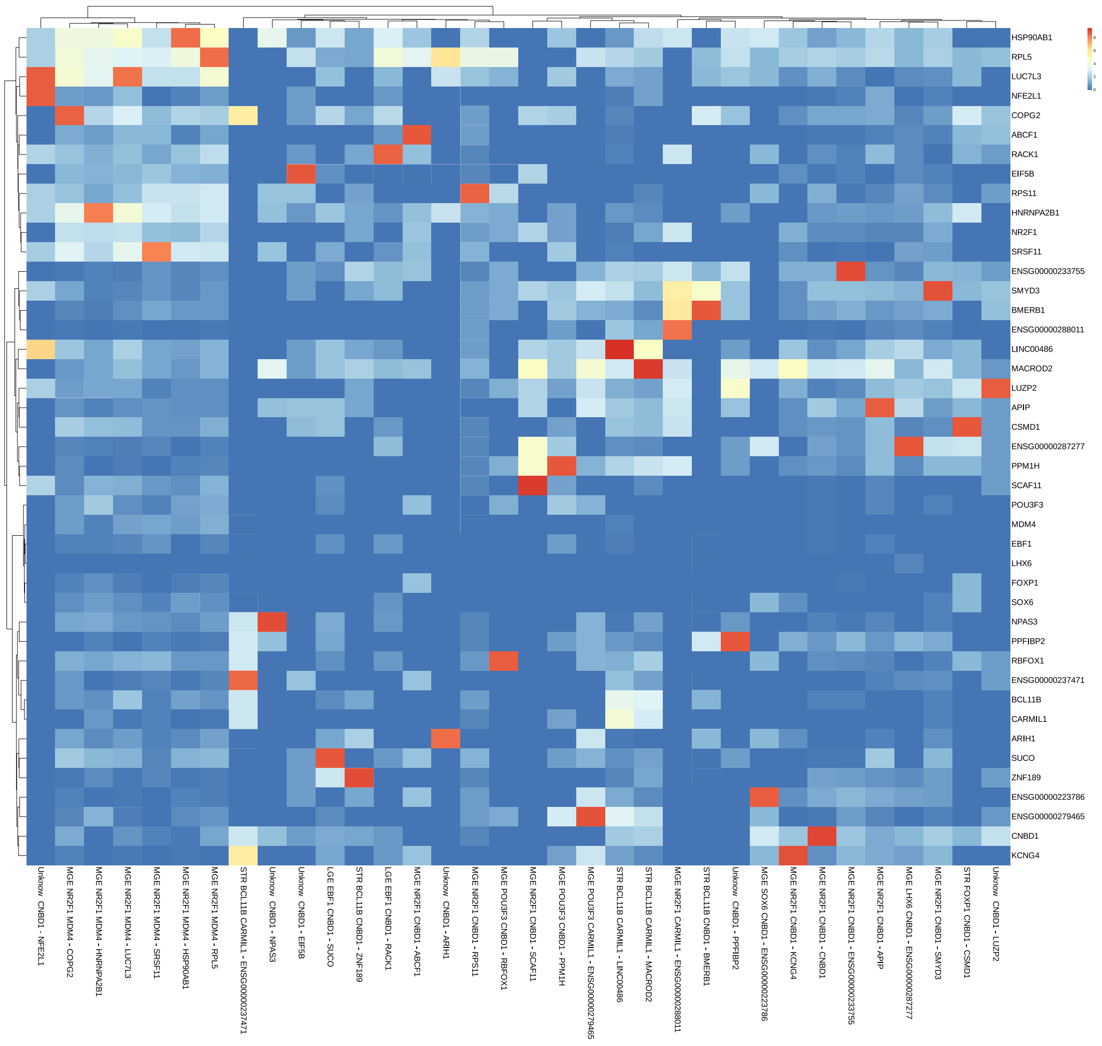


HDMAP [High Density Approximation and Projection] plot:



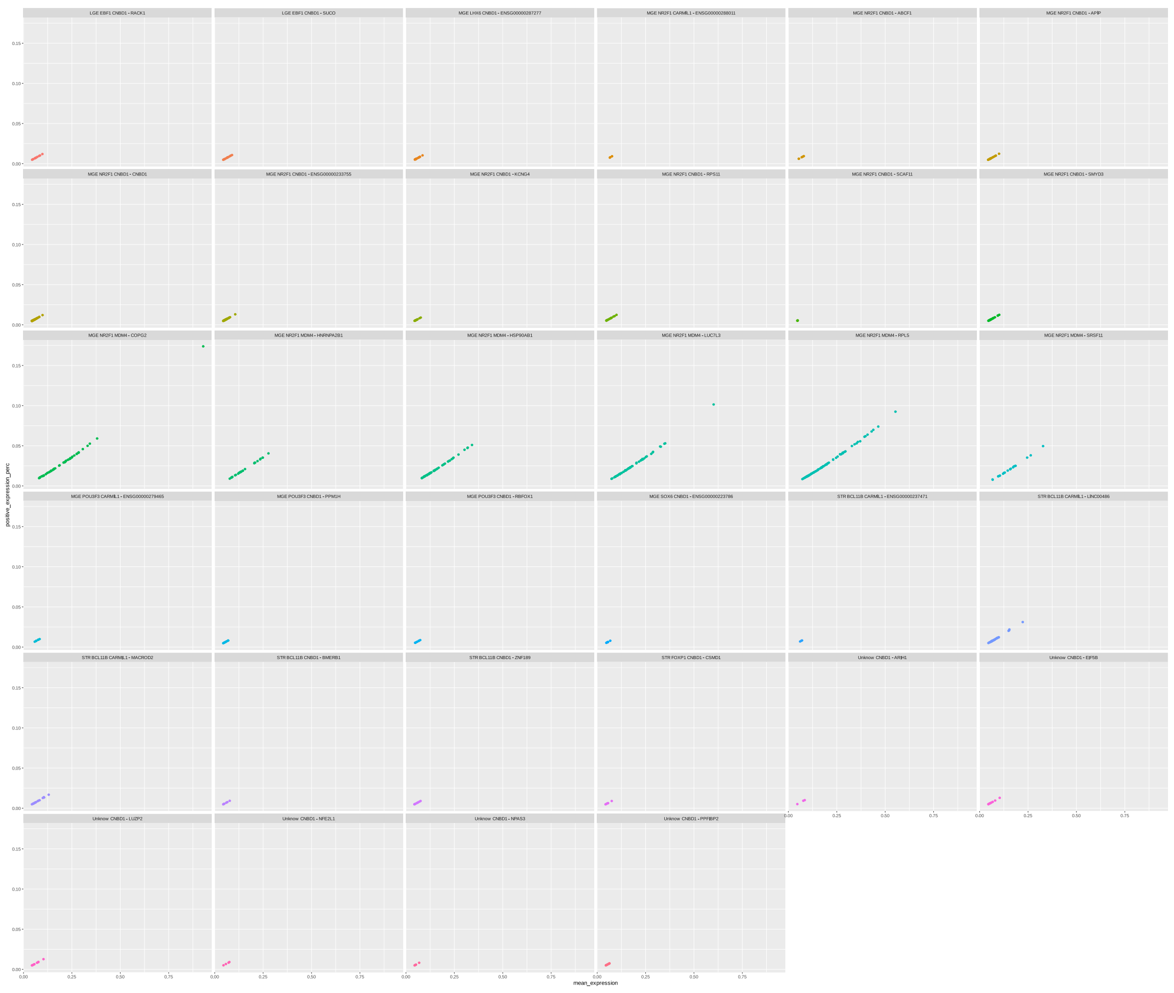
The HDMAP was developed for better visualization of obtained cell subtypes by cluster subsetting using the CSSG algorithm results. This approach is based on UMAP calculation with additional steps. The HDMAP is based on paternal clusters UMAP components 1 and 2 coordinates, which are averaged as centroids of these clusters. Next, the centroids information for each cell is multiplied by a factor of 1000 for increasing the density of co-clusters, which will be based on paternal UMAP cluster centroids. After that for each cluster is computed UMAP using all gene combination results from the CSSG algorithm where *loss_pval* <= Q25 for the given cluster. Finally, information from centroids is summed with new UMAP 1 and 2 components for each cluster information and plotted.

Heatmap:



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

Scatter plot for ratio of number of genes and genes level of expression:



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

8. 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

fastp

https://github.com/OpenGene/fastp

Seurat

https://github.com/satijalab/seurat

UMI-tools

https://github.com/CGATOxford/UMI-tools

UMAP

https://umap-learn.readthedocs.io/en/latest/

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