

Pseudo-temporal ordering and RNA velocity – Practical session

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Workshop

**The
Hitchhiker's
Guide
to scRNA-seq**

Outlines

- **Introduction**
 - Setup your environment (R & Python)
 - The example data set
- **Analysis**
 - Brief overview on the data (R)
 - Diffusion pseudotime and trajectory analysis (R)
 - Data conversion (R & Python)
 - Diffusion pseudotime (Python)
 - Coarse-grained trajectory analysis with PAGA (Python)
 - RNA velocity analysis with scVelo (Python)
 - Fate probability estimation with CellRank 2 (Python)

Set up your conda environment

Packages in need:

- R
 - Seurat
 - reticulate
 - anndata
 - destiny
 - URD
- Python
 - scanpy
 - scvelo
 - cellrank
 - (velocity.py)

Set up your conda environment (Linux/MacOS users):

```
> conda create -n env_hitchhiker2024 python=3.9 r-base=4 jupyterlab r-reticulate r-irkernel r-devtools  
scanpy scvelo cellrank python-igraph r-Seurat=5 cython gsl udunits2 -c conda-forge --solver=libmamba  
> conda activate env_hitchhiker2024  
> conda install -c bioconda -c conda-forge velocity.py r-anndata  
(Linux) > conda install -c conda-forge gcc gxx  
(Linux) > conda install -c bioconda -c conda-forge bioconductor-destiny  
(MacOS/Linux-failed) > conda install -c conda-forge r-biocmanager cmake  
(MacOS/Linux-failed) > echo 'BiocManager::install("destiny")' | R --vanilla  
> echo 'devtools::install_github("farrellja/URD")' | R --vanilla  
> echo 'devtools::install_github("mojaveazure/seurat-disk")' | R --vanilla
```

Compilation tools for MacOS users

<https://mac.r-project.org/tools/>

More for MacOS (ARM64) users:

At terminal, do

```
> conda activate env_hitchhiker2024  
> open `which R | sed 's/bin\/R$/lib\/R\/etc\/Makeconf/'`
```

This will open the TextEdit app. Look for the line starting with CPPFLAGS. Add the following content to the end of the line: `-DHAVE_WORKING_LOG1P`

Next, save and close the file

R package compilation environment for Win users
<https://cran.r-project.org/bin/windows/Rtools/>

Alternative option for Win users
Install a Linux environment with WSL2

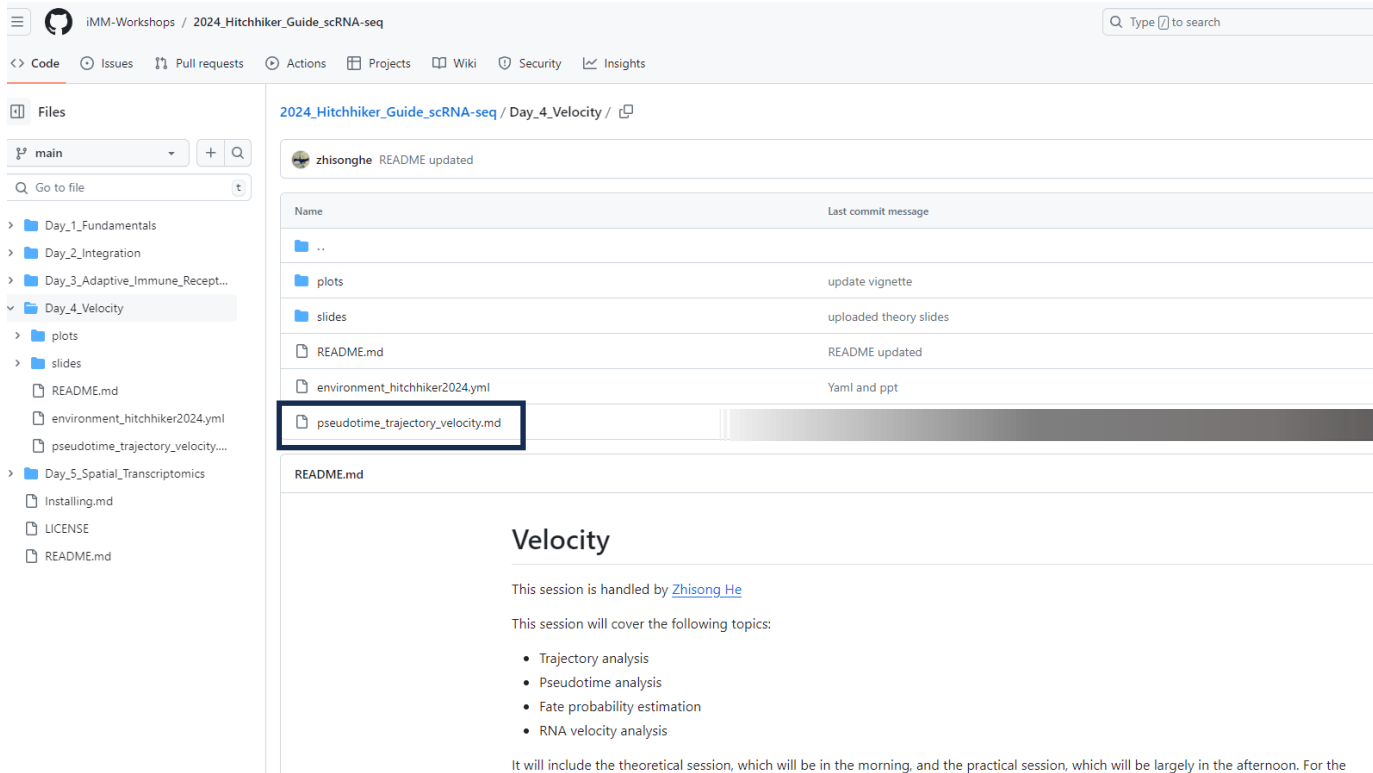
More information can be seen here:

https://github.com/iMM-Workshops/2024_Hitchhiker_Guide_scRNA-seq/blob/main/Day_4_Velocity/README.md

(Also includes non-conda way of setting up the environment)

Online vignette for the analysis

https://github.com/iMM-Workshops/2024_Hitchhiker_Guide_scRNA-seq/blob/main/Day_4_Velocity/



2024_Hitchhiker_Guide_scRNA-seq / Day_4_Velocity /

zhisonghe README updated

Name	Last commit message
..	
plots	update vignette
slides	uploaded theory slides
README.md	README updated
environment_hitchhiker2024.yml	Yaml and ppt
pseudotime_trajectory_velocity.md	

README.md

Velocity

This session is handled by [Zhisong He](#)

This session will cover the following topics:

- Trajectory analysis
- Pseudotime analysis
- Fate probability estimation
- RNA velocity analysis

It will include the theoretical session, which will be in the morning, and the practical session, which will be largely in the afternoon. For the

(R) Load the data and check information

Load the Seurat object and read in the object

```
library(Seurat)
seurat <- readRDS("DS1.rds")

Loading required package: SeuratObject
Loading required package: sp

Attaching package: 'SeuratObject'

The following object is masked from 'package:base':
  intersect
```

Check the basic information of the data

```
dim(seurat)
head(seurat@meta.data)
names(seurat@reductions)
```

<style>.list-inline {list-style: none; margin: 0; padding: 0; .list-inline>li {display: inline-block; .list-inline>li:not(:last-child):after {content: "\00b7"; padding: 0 .5ex}}</style>
1. 33694
2. 4317

A data.frame: 6 x 6


	orig.ident	nCount_RNA	nFeature_RNA	percent.mt	celltype	region
	<fct>	<dbl>	<int>	<dbl>	<fct>	<fct>
AAACCTGAGAATGTG-1	DS1	16752	3287	1.098376	G2M Dten. and midbrain NPC	Non-telencephalon
AAACCTGAGAGCCTAG-1	DS1	13533	3399	1.950787	G2M Dten. and midbrain NPC	Non-telencephalon
AAACCTGAGTAATCCC-1	DS1	3098	1558	2.453196	Midbrain-hindbrain boundary neuron	Non-telencephalon
AAACCTGCACACTGCG-1	DS1	5158	2015	3.761148	Midbrain hindbrain boundary neuron	Non-telencephalon
AAACCTGCATCGGAAG-1	DS1	6966	2322	2.182027	Dorsal telen. IP	Dorsal telencephalon
AAACCTGGTGTAACGG-1	DS1	4108	1542	2.507303	Unknown 1	NA

<style>.list-inline {list-style: none; margin: 0; padding: 0; .list-inline>li {display: inline-block; .list-inline>li:not(:last-child):after {content: "\00b7"; padding: 0 .5ex}}</style>
1. 'pca'
2. 'umap'
3. 'tsne'

Feature plots to better understand the data

Alternative vignette for the analysis (R/Seurat-centric)

https://github.com/quadbio/scRNAseq_analysis_vignette



scRNAseq_analysis_vignette

Public

Edit Pins

Unwatch 5

Fork 41

Starred 98

master


1 Branch

0 Tags

Go to file

Add file

Code

 Zhisong He

tutorial updated

bb4bc82 · 2 years ago

34 Commits

data	added cell communication analysis	3 years ago
images	tutorial updated, velocity pseudotime	2 years ago
README.md	label transfer added	2 years ago
Tutorial.md	Tutorial updated	2 years ago
Tutorial.pdf	Tutorial updated	2 years ago

README

Tutorial for scRNA-seq data analysis beginners using R

This tutorial includes three different parts:

1. The most basic and routine analysis on one scRNA-seq data set using `Seurat` in R;
2. Data integration or batch effect correction for joint analysis of multiple scRNA-seq data sets;
3. Cell type annotation label transfer given the reference data set;
4. Brief introduction of more advanced analysis, including velocity analysis and ligand-receptor-pairing-based cell-cell communication analysis.

The example data used in this tutorial are mostly from the paper [Organoid single-cell genomic atlas uncovers human-specific features of brain development](#). In addition, a subset of data presented in the paper [Charting human development using a multi-endodermal organ atlas and organoid models](#) is also included as the example data set for ligand-receptor pairing analysis.

Please contact Dr. Zhisong He ([zhisong.he\(at\)bsse.ethz.ch](mailto:zhisong.he(at)bsse.ethz.ch)) or Prof. Barbara Treutlein ([barbara.treutlein\(at\)bsse.ethz.ch](mailto:barbara.treutlein(at)bsse.ethz.ch)) if there is any question.

About

Tutorial for scRNA-seq data analysis beginners using R

bioinformatics

tutorial

single-cell-rna-seq

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Contributors 2

 quadbiolab

QuaDBio Lab (archived)

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Zhisong He

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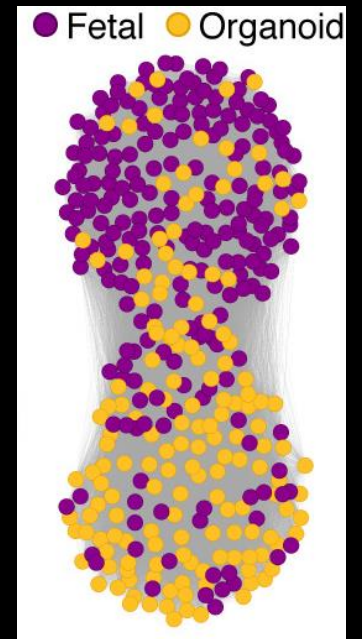
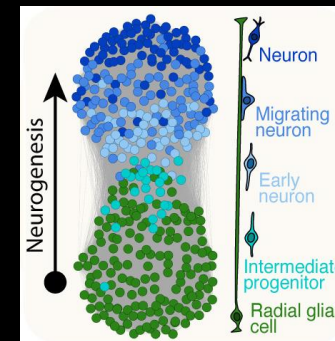
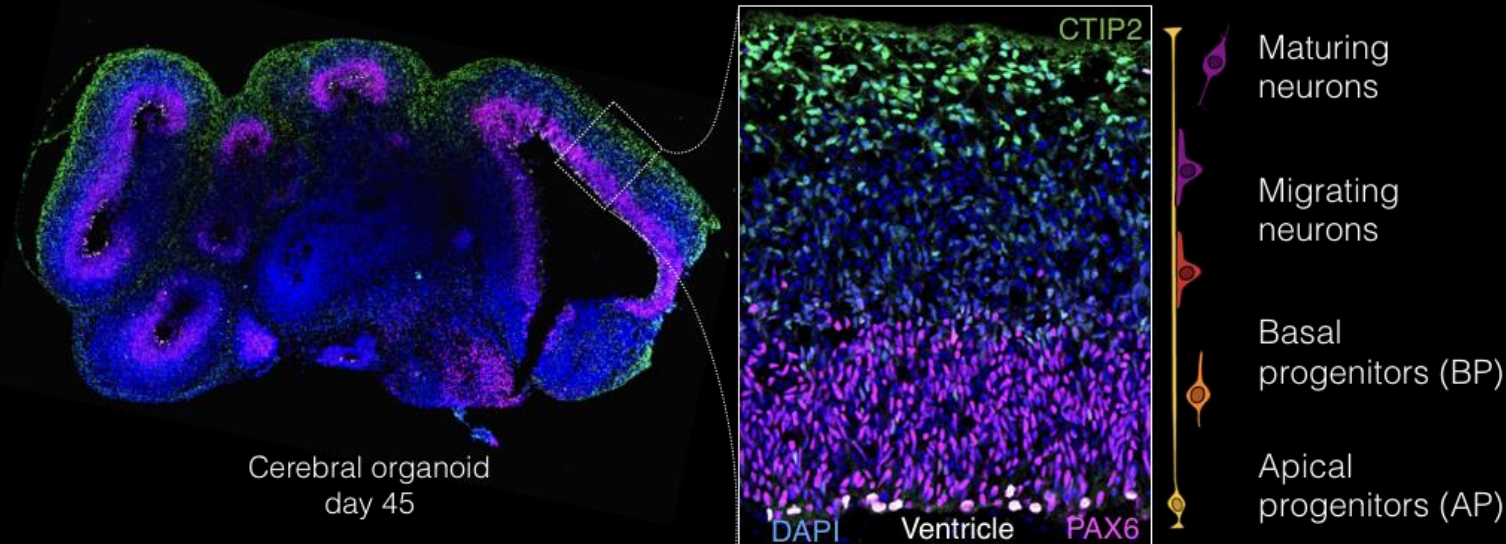
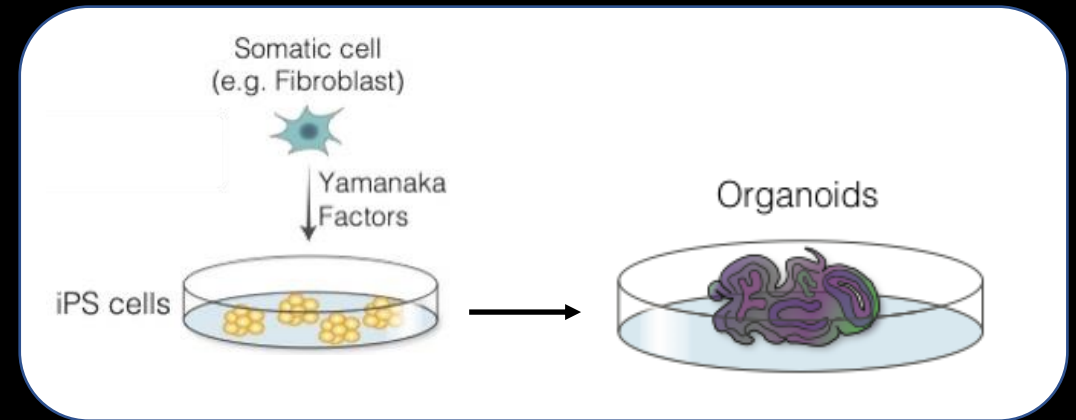
Example data set: scRNA-seq data of brain organoids

LETTER

<https://doi.org/10.1038/s41586-019-1654-9>

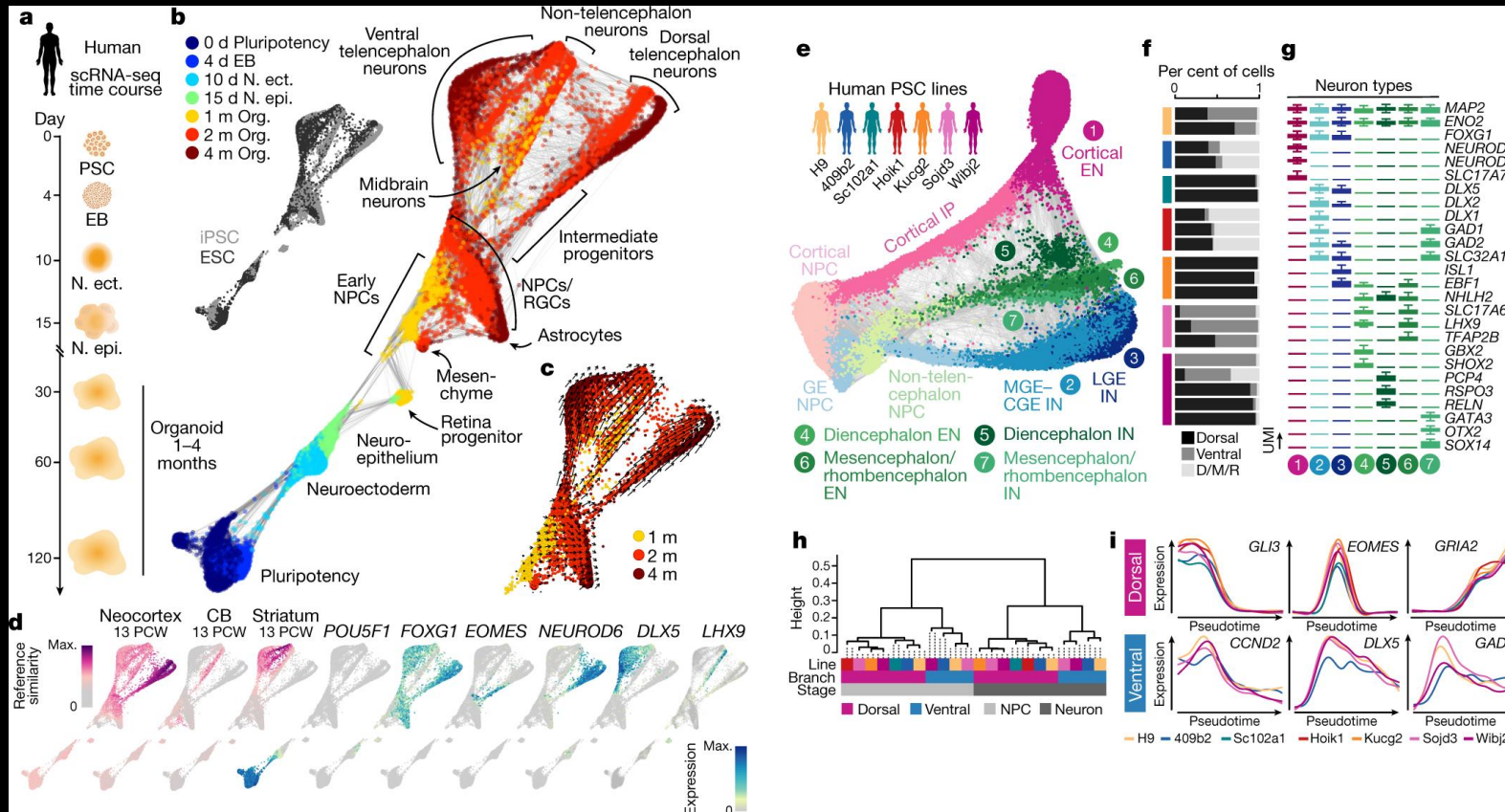
Organoid single-cell genomic atlas uncovers human-specific features of brain development

Sabina Kanton^{1,7}, Michael James Boyle^{1,7}, Zhisong He^{1,2,7*}, Malgorzata Santel¹, Anne Weigert¹, Fátima Sanchís-Calleja^{1,2}, Patricia Guijarro³, Leila Sidow¹, Jonas Simon Fleck², Dingding Han³, Zhengzong Qian³, Michael Heide⁴, Wieland B. Huttner⁴, Philipp Khaitovich^{1,3,5}, Svante Pääbo¹, Barbara Treutlein^{1,2*} & J. Gray Camp^{1,6*}



Camp et al. (2015) Human cerebral organoids recapitulate gene expression programs of fetal neocortex development. *PNAS*

Example data set: scRNA-seq data of brain organoids



● Example data set

Example data set

One 2-month-old organoid

- 4317 cells
- Seurat object
- Preprocessed and annotated
- Also include exonic/intronic count matrices as additional assays

Link to the data (RDS file for the Seurat object):
<https://polybox.ethz.ch/index.php/s/bjNnfD9l3rwpjlt>

Link to the data (H5AD file for the AnnData object):
<https://polybox.ethz.ch/index.php/s/bUYZE6qPgROBggH>

Outlines

- **Introduction**
 - Setup your environment (R & Python)
 - The example data set
- **Analysis**
 - Brief overview on the data (R) – 5 min
 - Diffusion pseudotime and trajectory analysis (R) – 20 min
 - Data conversion (R & Python) – 5 min
 - Diffusion pseudotime (Python) – 10 min
 - Coarse-grained trajectory analysis with PAGA (Python) – 10 min
 - RNA velocity analysis with scVelo (Python) – 20 min
 - Fate probability estimation with CellRank 2 (Python) – 20 min

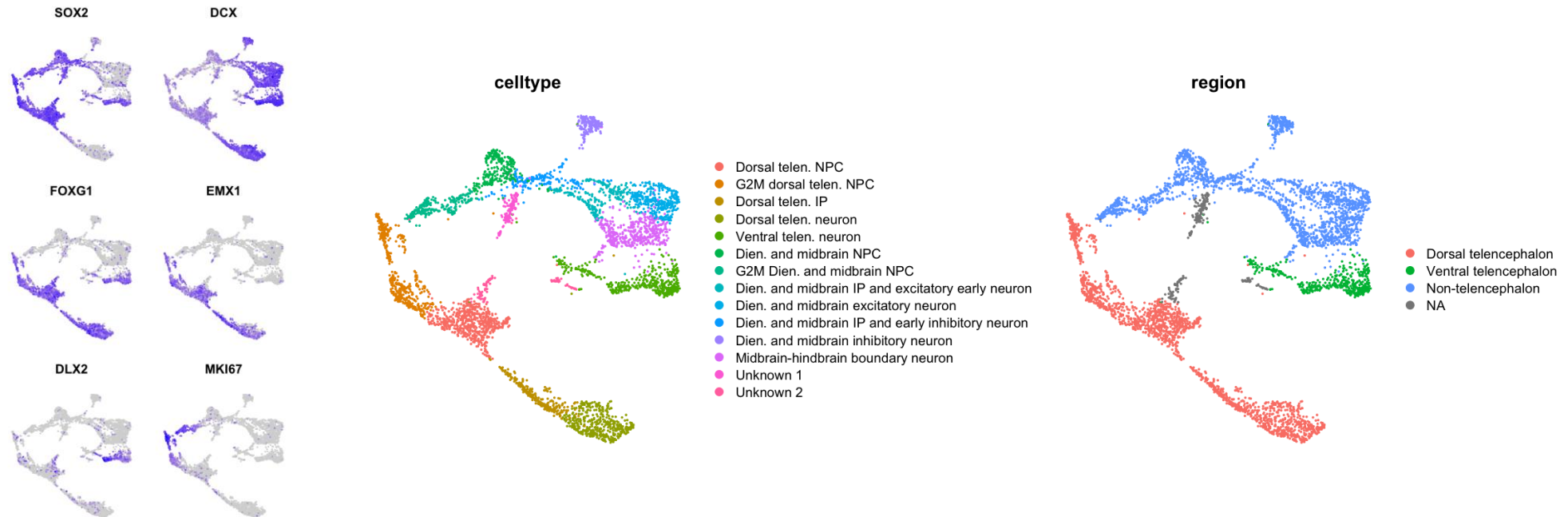
Brief overview on the data

```
> library(Seurat)

> seurat <- readRDS('DS1.rds')

> dim(seurat)
> head(seurat@meta.data)
> names(seurat@reductions)

> FeaturePlot(seurat, c('SOX2','DCX','FOXG1','EMX1','DLX2','MKI67'), order=T) & NoAxes() & NoLegend()
> p1 <- UMAPPlot(seurat, group.by=c('celltype')) & NoAxes()
> p2 <- UMAPPlot(seurat, group.by=c('region')) & NoAxes()
> p1 | p2
```

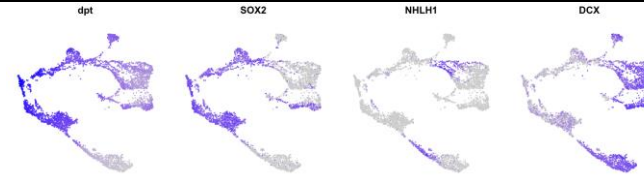


Diffusion map in R

```
> library(density)

> seurat <- subset(seurat, subset = celltype %in% setdiff(levels(seurat$celltype),
c('Unknown 1','Unknown 2')))
> seurat <- RunPCA(seurat, npcs=20)
> dm <- DiffusionMap(Embeddings(seurat, "pca")[,1:20], k=50)
> dpt <- DPT(dm)
> seurat$dpt <- rank(dpt$dpt)
> FeaturePlot(seurat, c("dpt","SOX2","NHLH1","DCX"), ncol=4) & NoAxes() & NoLegend()
```

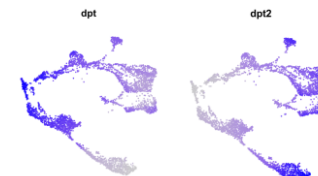
Run DPT with default parameters



```
> tips_cand <- sapply(1:100, function(i){ random_root(dm) })
> idx_NPC <- which(seurat@meta.data$celltype %in% c('Dorsal telen. NPC',
                                                    'G2M dorsal telen. NPC',
                                                    'Dien. and midbrain NPC',
                                                    'G2M Dien. and midbrain NPC'))
> tips_cand <- as.numeric(names(which.max(table(tips_cand[tips_cand %in% idx_NPC]))))
> dpt2 <- DPT(dm, tips=tips_cand)
> seurat$dpt2 <- rank(dpt2$dpt)

> FeaturePlot(seurat, c("dpt","dpt2"), ncol=2) & NoAxes() & NoLegend()
```

Run DPT with default parameters, while ensuring
a progenitor cell is chosen as the tip

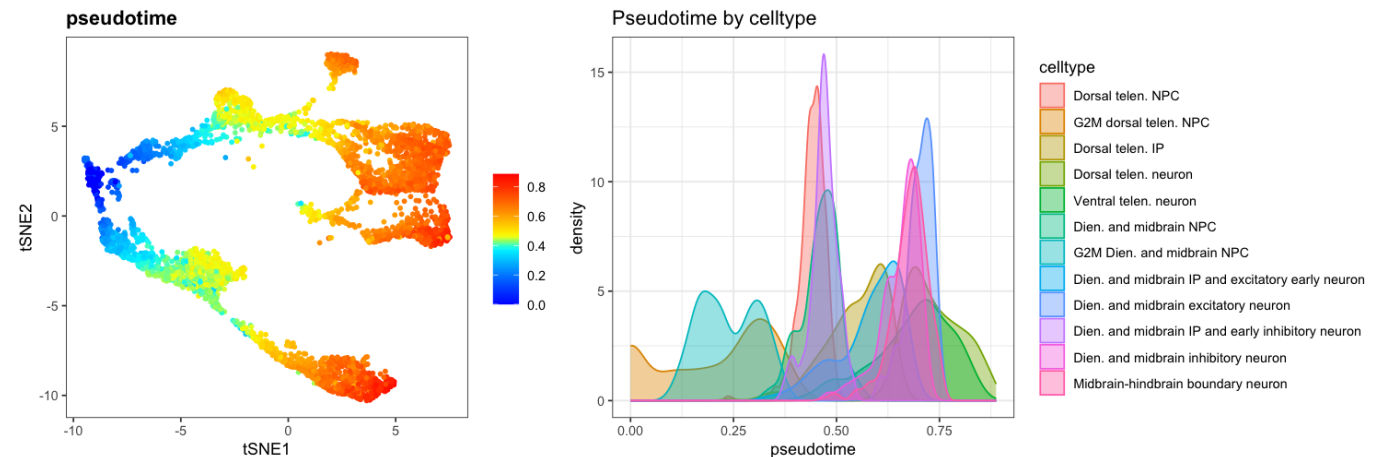


(Optional) Trajectory analysis in R with URD

```
> library(URD)

> urd <- createURD(count.data = seurat[['RNA']]@counts, meta=seurat@meta.data,
min.cells=0, min.counts=0)
> urd@pca.scores <- as.data.frame(Embeddings(seurat,'pca'))
> urd@tsne.y <- setNames(as.data.frame(Embeddings(seurat,'umap')), c('tSNE1','tSNE2'))
> urd@dm <- dm
> root_cells <- colnames(seurat)[order(seurat$dpt2)[1:100]]
> floods <- floodPseudotime(urd, root.cells = root_cells, n=50, minimum.cells.flooded =
2, verbose=F)
> urd <- floodPseudotimeProcess(urd, floods, floods.name="pseudotime")
> root_cells <- rownames(urd@meta)[order(urd@pseudotime$pseudotime)[1:50]]
> p1 <- plotDim(urd, "pseudotime")
> p2 <- plotDists(urd, "pseudotime", "celltype", plot.title="Pseudotime by celltype")
> p1 | p2
```

Get flood diffusion based pseudotime
with URD



(Optional) Trajectory analysis in R with URD (2)

```
> seurat@meta.data$fpt <- urd@pseudotime$pseudotime
> cor(seurat$fpt, seurat$dpt2, method='spearman')
> plot(seurat$fpt, seurat$dpt2, pch=16, col='#30303050', frame=F)
```

Compare URD-based pseudotime with DPT

```
> neuron_types <- setdiff(grep('neuron', levels(seurat$celltype), value=T), grep('early',
levels(seurat$celltype), value=T))
> idx_tips <- unlist(lapply(neuron_types, function(x){
  which(seurat$celltype==x)[order(seurat$fpt[seurat$celltype == x], decreasing=T)[1:50]]
}))
> urd@group.ids[idx_tips, 'tip.clusters'] <- as.numeric(droplevels(seurat$celltype[idx_tips]))
```

Assign tip populations

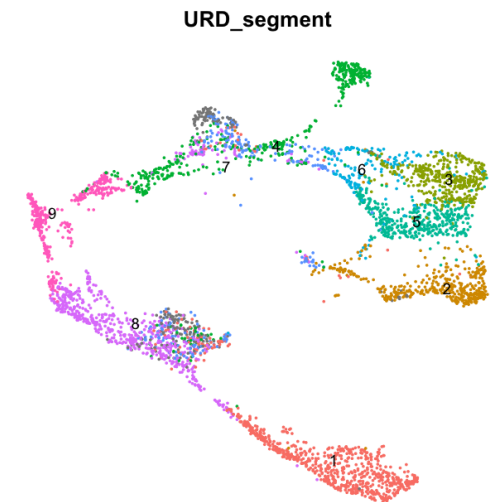
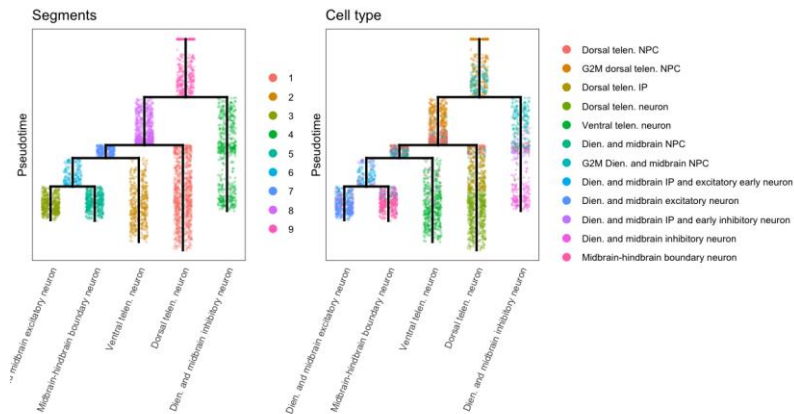
```
> ptlogistic <- pseudotimeDetermineLogistic(urd, "pseudotime", optimal.cells.forward=20,
max.cells.back=20, do.plot = T)
> biased.tm <- as.matrix(pseudotimeWeightTransitionMatrix(urd, "pseudotime",
logistic.params=ptlogistic))
> walks <- simulateRandomWalksFromTips(urd, tip.group.id = "tip.clusters", root.cells = root_cells,
transition.matrix = biased.tm, n.per.tip = 5000, root.visits = 1, max.steps = 4000, verbose = F)
> urd <- processRandomWalksFromTips(urd, walks, verbose = F)
```

Random walk from roots to tips

(Optional) Trajectory analysis in R with URD (3)

```
> tree <- loadTipCells(urd, "tip.clusters")
> tree <- buildTree(tree, pseudotime = "pseudotime", tips.use=NULL, divergence.method = "preference",
cells.per.pseudotime.bin = 25, bins.per.pseudotime.window = 8, save.all.breakpoint.info = T,
p.thresh=0.001)
> tree <- nameSegments(tree, segments= sort(unique(tree@group.ids$tip.clusters)), segment.names =
levels(droplevels(seurat$celltype[idx_tips])), short.names = c('dTN','vTN','DMExN','DMInN','MHBN'))
>
> p1 <- plotTree(tree, "segment", title="Segments")
> p2 <- plotTree(tree, "celltype", title="Cell type")
> p1 | p2
```

Build and visualize the differentiation tree



```
> seurat@meta.data$URD_segment <- tree@group.ids$segment
> UMAPplot(seurat, group.by='URD_segment', label=T) & NoAxes() & NoLegend()
```

Data conversion from Seurat to AnnData (h5ad)

```
> library(anndata)
> library(Matrix)
> shared_genes <- intersect(rownames(seurat[['RNA']]),
                           intersect(rownames(seurat[['spliced']]),
                                    rownames(seurat[['unspliced']])))
> adata <- AnnData(X = t(seurat[['RNA']]@data[shared_genes,]),
                  obs = seurat@meta.data,
                  var = seurat[['RNA']]@meta.features[shared_genes,],
                  layers = list(counts = t(seurat[['RNA']]@counts[shared_genes,]),
                                spliced = t(seurat[['spliced']]@counts[shared_genes,]),
                                unspliced = t(seurat[['unspliced']]@counts[shared_genes,])),
                  obsm = list(X_pca = Embeddings(seurat,"pca")[,1:20],
                              X_umap = Embeddings(seurat,"umap"))
                  )
> adata$write_h5ad("DS1.h5ad")
```

P.S. *SeuratDisk* (<https://github.com/mojaveazure/seurat-disk>) also provides the Seurat to h5ad conversion functionality. However, it designs to work for only one Assay, for which it converts the "data" slot/layer of the Array into the "X" slot of the AnnData, and the "counts" slot/layer into a matrix in the "layers" slot of the AnnData. This doesn't work for what we want to do here

P.S. In an AnnData object, it is required that all data matrices (X and all matrices in the "layer" slot) share the same dimensionalities. Therefore, we have to subset into genes appear in all the three matrices.

Diffusion map in Python

```
>>> import scanpy as sc
>>> adata = sc.read_h5ad('DS1.h5ad')
>>> sc.pp.neighbors(adata, n_neighbors=50, n_pcs=20, use_rep='X_pca')
>>> sc.tl.diffmap(adata, n_comps=20)
```

Run diffusion map

P.S. In *scanpy*, the diffusion pseudotime (*scanpy.tl.dpt*) function requires the root cell to be manually labeled. To use the same root guessing procedure as implemented in *destiny* in R, we have to re-implement it with the following code:

```
import random
import numpy as np
import pandas as pd
def random_root(adata, seed = None, neighbors_key=None, idx_subset = None):
    if seed is not None:
        random.seed(seed)
    iroot_bak = None
    if 'iroot' in adata.uns.keys():
        iroot_bak = adata.uns['iroot'].copy()
    dpt_bak = None
    if 'dpt_pseudotime' in adata.obs.columns:
        dpt_bak = adata.obs['dpt_pseudotime'].copy()

    idx = np.random.choice(list(range(adata.shape[0])))
    adata.uns['iroot'] = idx
    sc.tl.dpt(adata, neighbors_key=neighbors_key)
    dpt = adata.obs['dpt_pseudotime']
    if idx_subset is not None:
        dpt = dpt.iloc[idx_subset]
    idx_max_dpt = np.argmax(dpt)
    if idx_subset is not None:
        idx_max_dpt = idx_subset[idx_max_dpt]

    del adata.uns['iroot']
    del adata.obs['dpt_pseudotime']
    if iroot_bak is not None:
        adata.uns['iroot'] = iroot_bak.copy()
    if dpt_bak is not None:
        adata.obs['dpt_pseudotime'] = dpt_bak.copy()

    return idx_max_dpt
```

Diffusion map in Python (2)

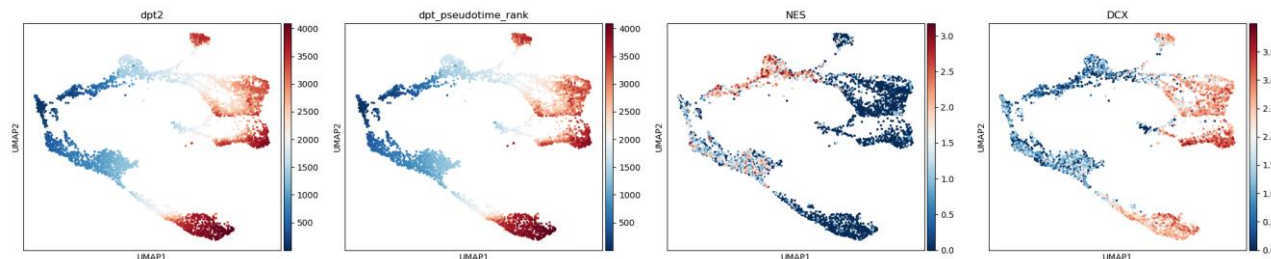
```
>>> idx_subset = np.where(np.isin(adata.obs['celltype'], ['Dorsal telen. NPC',  
                                                         'G2M dorsal telen. NPC',  
                                                         'Dien. and midbrain NPC',  
                                                         'G2M Dien. and midbrain NPC']))[0]  
>>> idxs_rand_root = np.apply_along_axis(lambda x: random_root(adata, idx_subset=idx_subset),  
                                         1, np.array(range(1000))[:,None])  
>>> adata.uns['iroot'] = np.argmax(np.bincount(idxs_rand_root))  
>>> sc.tl.dpt(adata, n_dcs=20)
```

Infer root cell among NPCs, and estimate diffusion pseudotimes

```
>>> from scipy.stats import pearsonr, spearmanr, rankdata  
>>> [pearsonr(adata.obs['dpt2'], adata.obs['dpt_pseudotime']),  
     spearmanr(adata.obs['dpt2'], adata.obs['dpt_pseudotime'])]  
  
>>> adata.obs['dpt_pseudotime_rank'] = rankdata(adata.obs['dpt_pseudotime'])  
>>> import matplotlib.pyplot as plt  
>>> plt.scatter(adata.obs['dpt2'], adata.obs['dpt_pseudotime_rank'])  
>>> plt.show()
```

Compare the Python-based DPT and R-based DPT

```
>>> sc.pl.umap(adata, color=['dpt2', 'dpt_pseudotime_rank', 'NES', 'DCX'], color_map='RdBu_r', ncols=4)
```

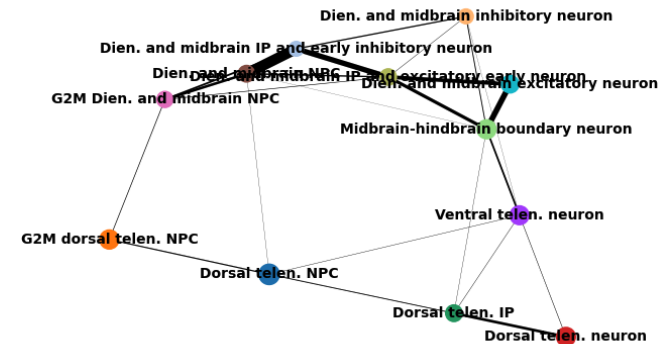


Coarse-grained trajectory analysis with PAGA

```
>>> adata.obs['celltype'] = adata.obs['celltype'].cat.remove_unused_categories()
>>> sc.pp.neighbors(adata, n_neighbors=20, n_pcs=20, use_rep='X_diffmap')
>>> sc.tl.paga(adata, groups='celltype')
>>> sc.pl.paga(adata)
```

Perform PAGA and visualize estimated cell type connectivities

	node1	node2
0	G2M dorsal telen. NPC	Dorsal telen. NPC
1	Dorsal telen. neuron	Dorsal telen. IP
2	G2M Dien. and midbrain NPC	Dien. and midbrain NPC
3	Dien. and midbrain IP and excitatory early neuron	Dien. and midbrain NPC
4	Dien. and midbrain excitatory neuron	Dien. and midbrain IP and excitatory early neuron
5	Dien. and midbrain IP and early inhibitory neuron	Dien. and midbrain NPC
6	Dien. and midbrain IP and early inhibitory neuron	G2M Dien. and midbrain NPC
7	Dien. and midbrain IP and early inhibitory neuron	Dien. and midbrain IP and excitatory early neuron
8	Dien. and midbrain inhibitory neuron	Dien. and midbrain IP and early inhibitory neuron
9	Midbrain-hindbrain boundary neuron	Ventral telen. neuron
10	Midbrain-hindbrain boundary neuron	Dien. and midbrain IP and excitatory early neuron
11	Midbrain-hindbrain boundary neuron	Dien. and midbrain excitatory neuron



```
>>> from scipy import sparse
>>> connected = adata.uns['paga']['connectivities'] > 0.1
>>> connected = (connected + connected.T) > 0
>>> idx_row, idx_col, dat = sparse.find(connected)
>>> idx = (idx_row >= idx_col)
>>> connected_celltypes = pd.DataFrame({ 'node1' : adata.obs['celltype'].cat.categories[idx_row[idx]],
                                         'node2' : adata.obs['celltype'].cat.categories[idx_col[idx]]})
>>> connected_celltypes
```

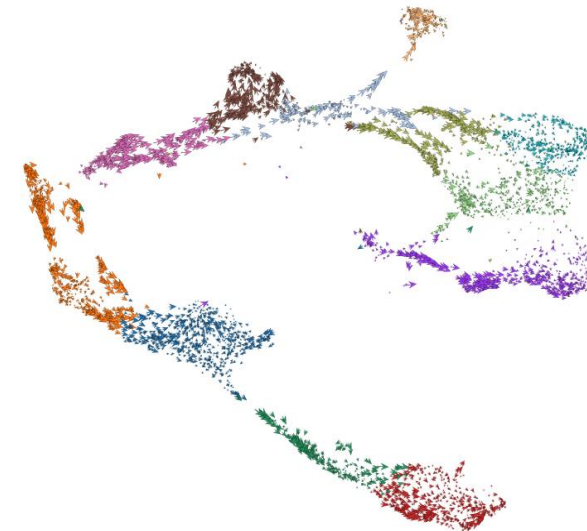
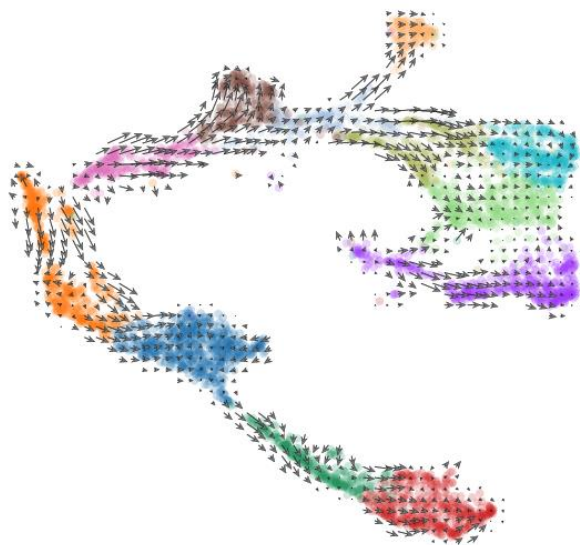
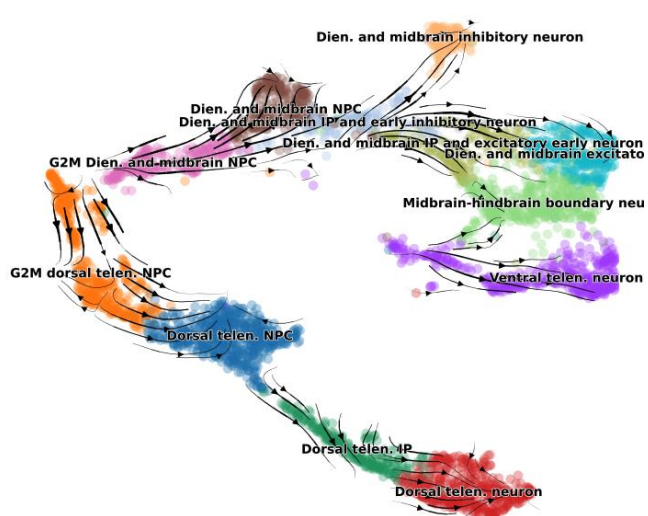
Check connected cell types

RNA velocity analysis with scVelo

```
>>> import scvelo as scv
>>> adata.raw = adata
>>> scv.pp.filter_and_normalize(adata,
                                min_shared_counts=10,
                                n_top_genes=3000)

>>> sc.pp.neighbors(adata, use_rep='X_pca')
>>> scv.pp.moments(adata, n_neighbors = None)
>>> scv.tl.velocity(adata, mode='stochastic')
>>> scv.tl.velocity_graph(adata)

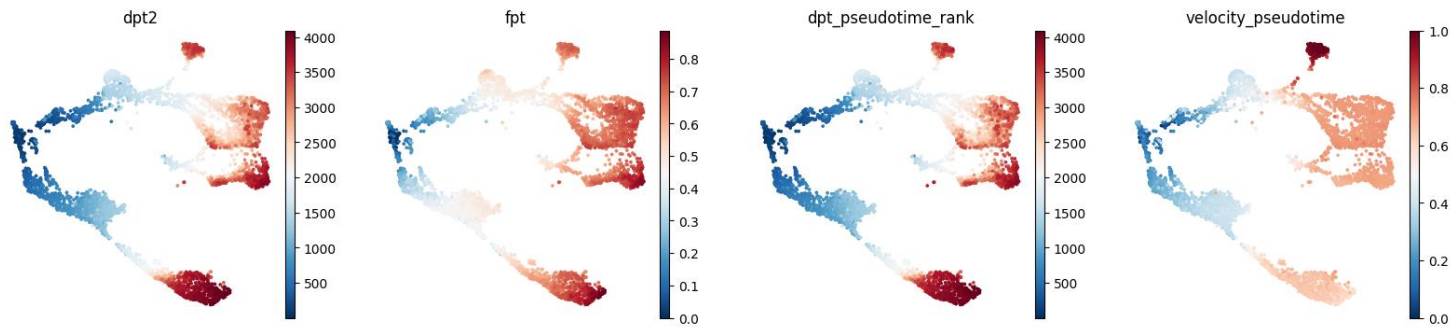
>>> scv.pl.velocity_embedding_stream(adata, basis="umap", color="celltype", frameon=False)
>>> scv.pl.velocity_embedding_grid(adata, basis="umap", color="celltype", frameon=False,
                                   arrow_size=2, arrow_length=2)
>>> scv.pl.velocity_embedding(adata, basis="umap", color="celltype", frameon=False,
                              arrow_size=2, arrow_length=2)
```



RNA velocity analysis with scVelo (2)

```
>>> scv.tl.velocity_pseudotime(adata)
>>> sc.pl.umap(adata, color=['dpt2', 'fpt', 'dpt_pseudotime_rank', 'velocity_pseudotime'],
cmap='RdBu_r', frameon=False, ncols=4)
```

Velocity-based pseudotime



Fate probability estimation with CellRank 2

```
>>> import cellrank as cr

>>> pk = cr.kernels.PseudotimeKernel(adata, time_key="dpt_pseudotime").compute_transition_matrix()
>>> ck = cr.kernels.ConnectivityKernel(adata).compute_transition_matrix()
>>> vk = cr.kernels.VelocityKernel(adata).compute_transition_matrix()

>>> combined_kernel = 0.5 * vk + 0.3 * pk + 0.2 * ck
```

Generate hybrid kernels summarizing velocity, transcriptomic similarity (connectivity) and pseudotime

```
>>> g = cr.estimators.GPCCA(combined_kernel)
>>> g.fit(n_states=15, cluster_key="celltype")
>>> g.predict_terminal_states(method="top_n", n_states=10)
>>> g.plot_macrostates(which="terminal")
```

Predict terminal states

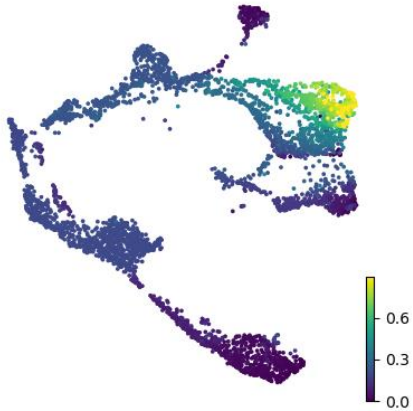
```
>>> g = cr.estimators.GPCCA(combined_kernel)
>>> neuron_types = [ x for x in adata.obs['celltype'].cat.categories if x.endswith('neuron') and
'early' not in x ]
>>> terminal_states =
[adata[adata.obs['celltype']==x,:].obs['dpt_pseudotime'].sort_values(ascending=False)[:30].index
for x in neuron_types]
>>> terminal_states = dict(zip(neuron_types, terminal_states))
>>> g.set_terminal_states(terminal_states)
>>> g.plot_macrostates(which="terminal")
```

Manually assign terminal states (each neuron type with the highest diffusion pseudotime)

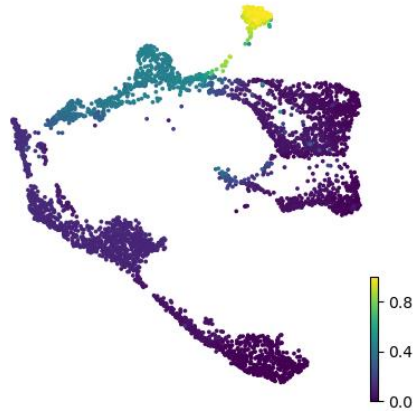
Fate probability estimation with CellRank 2 (2)

```
>>> g.compute_fate_probabilities()  
>>> g.plot_fate_probabilities(legend_loc="right", basis='X_umap', same_plot=False)
```

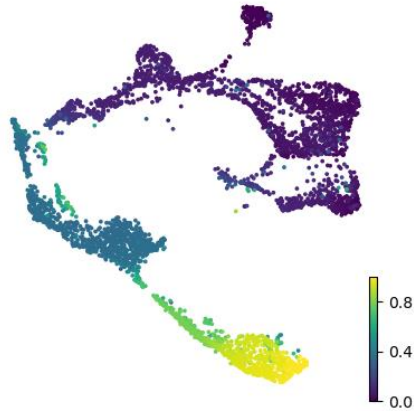
fate probabilities Dien. and midbrain excitatory neuron



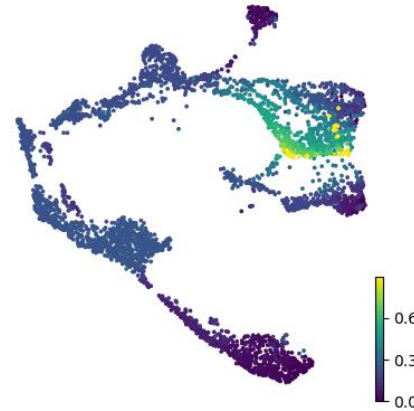
fate probabilities Dien. and midbrain inhibitory neuron



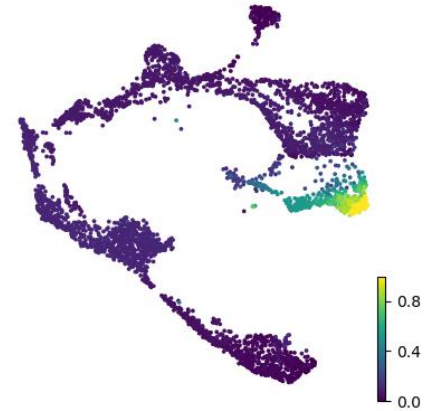
fate probabilities Dorsal telen. neuron



fate probabilities Midbrain-hindbrain boundary neuron



fate probabilities Ventral telen. neuron



Questions?