```
from scipy.io import mmread
import numpy as np
import pandas as pd
import scanpy as sc
import matplotlib.pyplot as plt
import seaborn as sns
import pymn
```

```
%matplotlib inline
```

```
#These save characters as text in PDFs
import matplotlib
matplotlib.rcParams['pdf.fonttype'] = 42
matplotlib.rcParams['ps.fonttype'] = 42

#These change plot aesthetics

sns.set(style='white', font_scale=1.25)
plt.rc("axes.spines", top=False, right=False)
plt.rc('xtick', bottom=True)
plt.rc('ytick', left=True)
```

Protocol 3: Functional characterization of replicating clusters

Protocol 3 demonstrates how to characterize functional gene sets contributing to cell type identity. Once replicating cell types have been identified with unsupervised MetaNeighbor (as in Protocols 1 and 2), supervised MetaNeighbor enables the functional interpretation of the biology contributing to each cell type's identity. In this protocol, we will focus on the characterization of inhibitory neuron subclasses from the mouse primary cortex as provided by the BICCN. The BICCN has shown that subclasses are strongly replicable across datasets and provided marker genes that are specific to each subclass. MetaNeighbor can be used to further quantify which pathways contribute to the subclasses' unique biological properties.

Step 1 - Creation of biologically relevant gene sets

1. To compute the functional characterization of clusters, we first need an ensemble of gene sets sampling relevant biological pathways. In this protocol we will consider the Gene Ontology (GO) annotations for mouse. The scripts used to build up-to-date gene sets can be found on Github, gene sets can be downloaded directly on FigShare.

```
!curl -L -o go_mouse.mtx https://ndownloader.figshare.com/files/24928064
!curl -L -o go_mouse_row_labels.txt https://ndownloader.figshare.com/files/24928067
```

```
!curl -L -o go_mouse_col_labels.txt https://ndownloader.figshare.com/files/24928061
```

```
  % Total    % Received % Xferd  Average Speed   Time    Time     Time  Current
                                 Dload  Upload   Total   Spent    Left  Speed
  0     0    0     0    0     0      0      0 --:--:-- --:--:-- --:--:--     0
100 20.9M  100 20.9M    0     0  7261k      0  0:00:02  0:00:02 --:--:-- 8582k
  % Total    % Received % Xferd  Average Speed   Time    Time     Time  Current
                                 Dload  Upload   Total   Spent    Left  Speed
  0     0    0     0    0     0      0      0 --:--:-- --:--:-- --:--:--     0
100  169k  100  169k    0     0   154k      0  0:00:01  0:00:01 --:--:--  154k
  % Total    % Received % Xferd  Average Speed   Time    Time     Time  Current
                                 Dload  Upload   Total   Spent    Left  Speed
  0     0    0     0    0     0      0      0 --:--:-- --:--:-- --:--:--     0
100 1207k  100 1207k    0     0   969k      0  0:00:01  0:00:01 --:--:-- 4890k
```

```
go_mtx = mmread('go_mouse.mtx')
go_genes = np.genfromtxt('go_mouse_row_labels.txt', dtype=str)
go_barcodes = np.genfromtxt('go_mouse_col_labels.txt',
                            dtype=str,
                            delimiter='\n')
```

```
go_sets = pd.DataFrame(go_mtx.todense(), index=go_genes, columns=go_barcodes)
```

Gene sets are stored as a one-hot encoded pandas dataframe. The index is genes, and columns are the GO terms, with 1s for when the gene is present in the set, and 0 otherwise.

1. Then we load our dataset containing inhibitory neurons from the BICCN. The scripts used to build the dataset can be found here, or downloaded directly from Figshare

```
!curl -L -o biccn_gaba.h5ad https://ndownloader.figshare.com/files/24928643
```

```
  % Total    % Received % Xferd  Average Speed   Time    Time     Time  Current
                                 Dload  Upload   Total   Spent    Left  Speed
  0     0    0     0    0     0      0      0 --:--:-- --:--:-- --:--:--     0
100  550M  100  550M    0     0  6744k      0  0:01:23  0:01:23 --:--:-- 6988k
```

```
biccn_gaba = sc.read_h5ad('biccn_gaba.h5ad')
```

```
biccn_gaba.obs.columns = biccn_gaba.obs.columns.astype(str)
```

```
Observation names are not unique. To make them unique, call `.obs_names_make_unique`.
```

```
biccn_gaba.obs['study_id'] = biccn_gaba.obs['study_id'].astype(str)
biccn_gaba.obs['joint_subclass_label'] = biccn_gaba.obs['joint_subclass_label'].astype(str)
```

1. Next we restrict our gene sets to genes that are present in the dataset. We then filter gene sets to keep gene sets of meaningful size: large enough to learn expression profiles (> 10), small enough to represent specific biological functions or processes (< 100).

```
go_sets.shape
```

```
(24403, 22546)
```

```
known_genes = biccn_gaba.var_names
shared_genes = np.intersect1d(known_genes, go_sets.index)
go_sets = go_sets.loc[shared_genes]
min_size = 10
max_size = 100
set_sizes = go_sets.sum()
go_sets = go_sets.loc[:, (set_sizes > min_size) & (set_sizes < max_size)]
```

```
go_sets.shape
```

```
(20536, 6066)
```

Step 2: Functional characterization with supervised MetaNeighbor

1. Once the gene set list is ready, we run the supervised *MetaNeighbor* function. Its inputs are similar to *MetaNeighborUS*, but it assumes that cell types have already been matched across datasets (i.e., they have identical names). Here we use joint BICCN subclasses, for which names have been normalized across datasets ("Pvalb", "Sst", "Sst Chodl", "Vip", "Lamp5", "Sncg"). Note that, because we are testing close to 6,500 gene sets, this step is expected to take a long time for large datasets. We recommend using this function inside a script and always save results to a file as soon as computations are done.

   PERFORMANCE NOTE 1 The `pymn.MetaNeighbor` function has 2 boolean parameters that control the speed and memory usage of the program. These parameters are `fast_version` and `fast_hi_mem`. The **slowest and highest memory** configuration is `fast_version=False fast_hi_mem=True`. The **lowest memory** configuration is `fast_version=True` and `fast_hi_mem=False`. To increase the speed and use a little bit more memory you can use `fast_version=True fast_hi_mem=True`. For the example below this requires about ~15GB of memory to run, but increases the speed from the **lowest memory** version by about 60%.

   PERFORMANCE NOTE 2 If you are running this on a computer/server with many cores/threads you might lose performance if you are using too many threads becasue of the overhead needed to start threads. You can control the number of threads using `import mkl; mkl.set_num_threads(n_threads)`. For our servers we found 16 threads to be the ideal amount. You can test it by running a few gene sets (10 or 100, `go_sets.iloc[:,:100]`) with different thread counts to see what the fastest is before running all the gene sets

```
import mkl; mkl.set_num_threads(16)
```

```
48
```

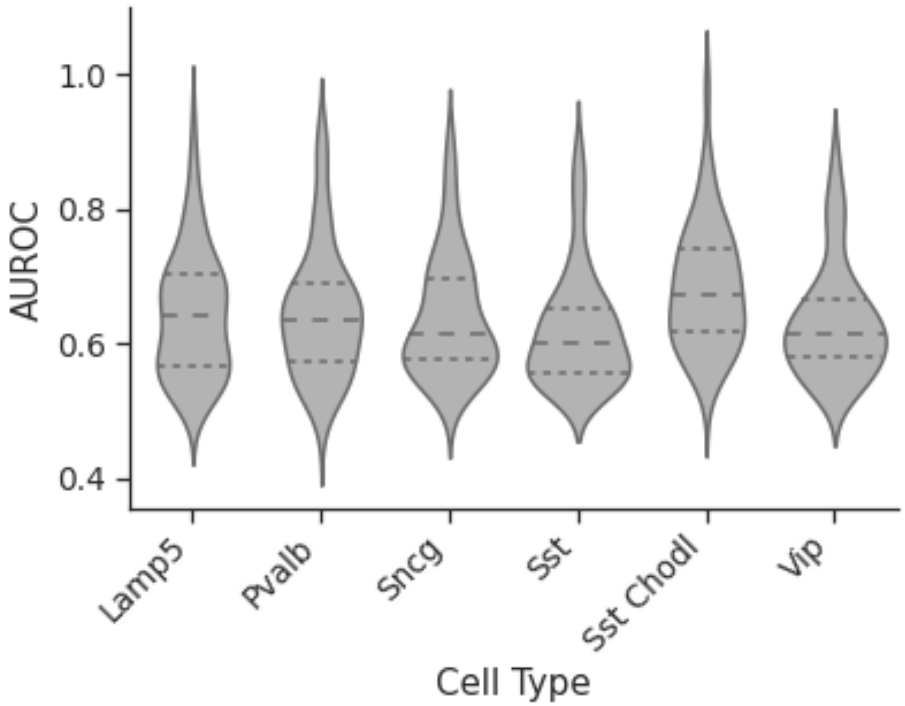
```
# pymn.MetaNeighbor(adata=biccn_gaba,
#                   study_col='study_id',
#                   ct_col='joint_subclass_label',
#                   genesets=go_sets,
#                   fast_version=True,
#                   fast_hi_mem=False)

# biccn_gaba.uns['MetaNeighbor'].to_csv('functional_aurocs.csv')
```

Later, results can be retrieved with the `pd.read_csv` function:

```
aurocs = pd.read_csv("functional_aurocs.csv", index_col=0)
biccn_gaba.uns['MetaNeighbor'] = aurocs
biccn_gaba.uns['MetaNeighbor_params'] = {'study_col':'study_id',
                                         'ct_col':'joint_subclass_label'}
```

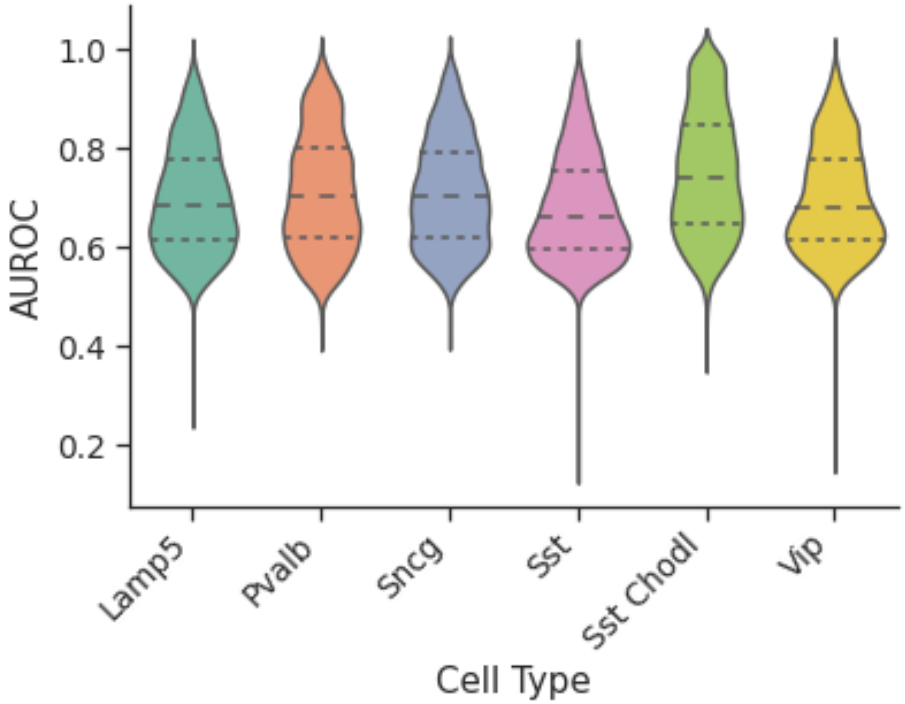
1. We use the "plotMetaNeighbor" function on the first 100 gene sets to rapidly visualize how replicability depends on gene sets.

```
pymn.plotMetaNeighbor(aurocs.iloc[:,:100])
```



If you pass the AnnData object with the results stored in `.uns` you can also color by cell-type if you want it to match other data. You can save a colormap as a dictionary of `{celltype:color}` under `adata.uns['{ct_col}_colors_dict']` to be consistent with your other plots, or let the program generate one from a given color palette under the `palette` parameter

```
pymn.plotMetaNeighbor(biccn_gaba, color='Cell Type')
```



In this representation, large the large dashed lines represent average gene set performance and the small dashed lines represent the 25 and 75 percent quantiles. We note that most gene sets contribute moderately to replicability (AUROC ~ 0.7), numerous gene sets have a performance close to random (AUROC ~ 0.5 - 0.6) and some gene sets have exceedingly high performance (AUROC > 0.8).

1. To focus on gene sets that contribute highly to specificity, we create a summary table containing, for each gene set, cell type specific AUROCs, average AUROC across all cell types and gene set size.

```
gs_size = go_sets.sum()
aurocs.loc['average_auroc'] = aurocs.mean()
aurocs.loc['gs_size'] = gs_size[aurocs.columns]
```

We then order gene set by AUROC and look at top scoring gene sets:

```
aurocs.T.sort_values('average_auroc', ascending=False).head(10)
```

Cell Type	Lamp5	Pvalb	\
GO:0007215|glutamate receptor signaling pathway|BP	0.967138	0.984908	
GO:0051966|regulation of synaptic transmission,...	0.959517	0.971045	
GO:0033555|multicellular organismal response to...	0.952360	0.975076	
GO:0060076|excitatory synapse|CC	0.960445	0.966509	
GO:0098839|postsynaptic density membrane|CC	0.924088	0.970252	
GO:0099565|chemical synaptic transmission, post...	0.968255	0.977103	
GO:0099601|regulation of neurotransmitter recep...	0.954515	0.974748	
GO:0060079|excitatory postsynaptic potential|BP	0.966917	0.976322	
GO:1900449|regulation of glutamate receptor sig...	0.953087	0.970946	
GO:0010771|negative regulation of cell morphoge...	0.979016	0.974905	

Cell Type	Sncg	Sst	\
GO:0007215|glutamate receptor signaling pathway|BP	0.970894	0.979208	
GO:0051966|regulation of synaptic transmission,...	0.975293	0.965359	
GO:0033555|multicellular organismal response to...	0.974724	0.947405	
GO:0060076|excitatory synapse|CC	0.988698	0.964131	
GO:0098839|postsynaptic density membrane|CC	0.983317	0.983514	
GO:0099565|chemical synaptic transmission, post...	0.972916	0.951548	
GO:0099601|regulation of neurotransmitter recep...	0.954839	0.951108	
GO:0060079|excitatory postsynaptic potential|BP	0.969234	0.950889	
GO:1900449|regulation of glutamate receptor sig...	0.952778	0.950950	
GO:0010771|negative regulation of cell morphoge...	0.970633	0.959184	

Cell Type	Sst Chodl	Vip	\
GO:0007215|glutamate receptor signaling pathway|BP	0.995992	0.986477	
GO:0051966|regulation of synaptic transmission,...	0.992332	0.973365	
GO:0033555|multicellular organismal response to...	0.999145	0.975227	
GO:0060076|excitatory synapse|CC	0.986668	0.957072	
GO:0098839|postsynaptic density membrane|CC	0.983329	0.970110	
GO:0099565|chemical synaptic transmission, post...	0.987180	0.955344	
GO:0099601|regulation of neurotransmitter recep...	0.993929	0.975712	
GO:0060079|excitatory postsynaptic potential|BP	0.986148	0.953739	
GO:1900449|regulation of glutamate receptor sig...	0.994219	0.972911	
GO:0010771|negative regulation of cell morphoge...	0.986028	0.924774	

Cell Type	average_auroc	gs_size
GO:0007215|glutamate receptor signaling pathway|BP	0.980770	92.0
GO:0051966|regulation of synaptic transmission,...	0.972819	75.0
GO:0033555|multicellular organismal response to...	0.970656	98.0
GO:0060076|excitatory synapse|CC	0.970587	75.0
GO:0098839|postsynaptic density membrane|CC	0.969101	93.0
GO:0099565|chemical synaptic transmission, post...	0.968724	91.0
GO:0099601|regulation of neurotransmitter recep...	0.967475	61.0
GO:0060079|excitatory postsynaptic potential|BP	0.967208	83.0
GO:1900449|regulation of glutamate receptor sig...	0.965815	56.0

```
GO:0010771|negative regulation of cell morphoge...       0.965757     98.0
```

Without surprise, replicability is mainly driven by gene sets related to neuronal functions that are immediately relevant to the physiology of inhibitory neurons, such as "glutamate receptor signaling pathway", "regulation of synaptic transmission, glutamatergic", or "chemical synaptic transmission, postsynaptic". Note that most of the top scoring gene sets have a large number of genes, as larger sets of genes make it easier to learn generalizable expression profiles. To obtain even more specific biological functions, we can further filter for gene sets that have fewer than 20 genes.

```
small_sets = aurocs.T[aurocs.T['gs_size'] < 20]
small_sets.sort_values('average_auroc', ascending=False).head(10)
```

```
Cell Type                                              Lamp5     Pvalb  \
GO:0004970|ionotropic glutamate receptor activi...  0.900527  0.917350
GO:0035235|ionotropic glutamate receptor signal...  0.822718  0.821832
GO:0032230|positive regulation of synaptic tran...  0.844397  0.856076
GO:0007216|G protein-coupled glutamate receptor...  0.889939  0.848807
GO:1905874|regulation of postsynaptic density o...  0.829720  0.862467
GO:0099150|regulation of postsynaptic specializ...  0.833023  0.892884
GO:0150052|regulation of postsynapse assembly|BP    0.833023  0.892884
GO:0021889|olfactory bulb interneuron different...  0.807886  0.913701
GO:0070679|inositol 1,4,5 trisphosphate binding|MF  0.920570  0.940097
GO:1902711|GABA-A receptor complex|CC               0.820995  0.868930

Cell Type                                              Sncg       Sst  \
GO:0004970|ionotropic glutamate receptor activi...  0.914096  0.957223
GO:0035235|ionotropic glutamate receptor signal...  0.905512  0.928740
GO:0032230|positive regulation of synaptic tran...  0.815510  0.920014
GO:0007216|G protein-coupled glutamate receptor...  0.763517  0.915839
GO:1905874|regulation of postsynaptic density o...  0.865795  0.903003
GO:0099150|regulation of postsynaptic specializ...  0.855117  0.909915
GO:0150052|regulation of postsynapse assembly|BP    0.855117  0.909915
GO:0021889|olfactory bulb interneuron different...  0.824044  0.883605
GO:0070679|inositol 1,4,5 trisphosphate binding|MF  0.790576  0.806067
GO:1902711|GABA-A receptor complex|CC               0.869553  0.801762

Cell Type                                          Sst Chodl       Vip  \
GO:0004970|ionotropic glutamate receptor activi...   0.966349  0.922562
GO:0035235|ionotropic glutamate receptor signal...   0.942784  0.866258
GO:0032230|positive regulation of synaptic tran...   0.984549  0.829219
GO:0007216|G protein-coupled glutamate receptor...   0.951083  0.842163
GO:1905874|regulation of postsynaptic density o...   0.916684  0.825713
GO:0099150|regulation of postsynaptic specializ...   0.909895  0.801576
GO:0150052|regulation of postsynapse assembly|BP     0.909895  0.801576
GO:0021889|olfactory bulb interneuron different...   0.891491  0.864433
```

```
GO:0070679|inositol 1,4,5 trisphosphate binding|MF   0.863020  0.852639
GO:1902711|GABA-A receptor complex|CC                0.990820  0.795980
```

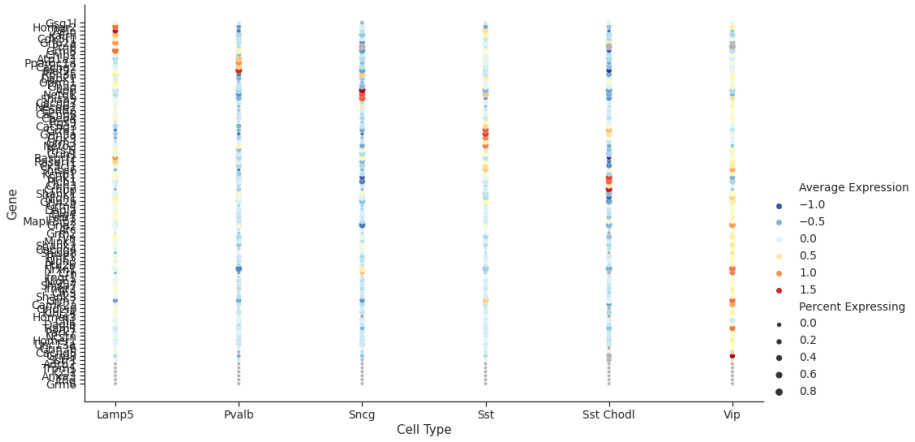
Cell Type	average_auroc	gs_size
GO:0004970|ionotropic glutamate receptor activi...	0.929684	19.0
GO:0035235|ionotropic glutamate receptor signal...	0.881307	16.0
GO:0032230|positive regulation of synaptic tran...	0.874961	16.0
GO:0007216|G protein-coupled glutamate receptor...	0.868558	16.0
GO:1905874|regulation of postsynaptic density o...	0.867230	19.0
GO:0099150|regulation of postsynaptic specializ...	0.867068	18.0
GO:0150052|regulation of postsynapse assembly|BP	0.867068	18.0
GO:0021889|olfactory bulb interneuron different...	0.864193	15.0
GO:0070679|inositol 1,4,5 trisphosphate binding|MF	0.862161	15.0
GO:1902711|GABA-A receptor complex|CC	0.858007	19.0

Again, the top scoring gene sets are dominated by biological functions immediately relevant to inhibitory neuron physiology, such as "ionotropic glutamate receptor signaling pathway", "positive regulation of synaptic transmission, GABAergic", or "GABA-A receptor complex".

1. To understand how individual genes contribute to gene set performance, we use the "plotDotPlot" function, which shows the expression of all genes in a gene set of interest, averaged over all datasets.

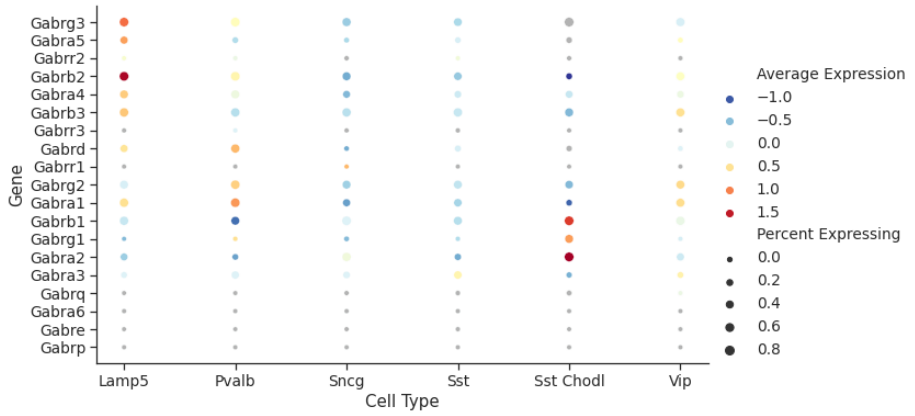
```
pymn.plotDotPlot(biccn_gaba,
                 go_sets["GO:0007215|glutamate receptor signaling pathway|BP"],
                 average_expressing_only=True,
                 fontsize=6,
                 figsize=(15, 9))
```

```
/home/bharris/miniconda3/lib/python3.7/site-packages/anndata/_core/anndata.py:1094: FutureWa
  if not is_categorical(df_full[k]):
/home/bharris/pyMN/pymn/plotting.py:127: RuntimeWarning: invalid value encountered in true_c
  axis=0) / np.nansum(M2, axis=1)
```




```
pymn.plotDotPlot(biccn_gaba,
                 go_sets["GO:1902711|GABA-A receptor complex|CC"],
                 average_expressing_only=True)
```

```
/home/bharris/miniconda3/lib/python3.7/site-packages/anndata/_core/anndata.py:1094: FutureWa
  if not is_categorical(df_full[k]):
/home/bharris/pyMN/pymn/plotting.py:127: RuntimeWarning: invalid value encountered in true_d
  axis=0) / np.nansum(M2, axis=1)
```



High scoring gene sets are characterized by the differential usage of genes from a given gene set. For example, when looking at the GABA-A receptor complex composition, Lamp5 preferentially uses the Gabrb2 and Gabrg3 receptors, Pvalb the Gabra1 receptor, and Sst Chodl the Gabra2, Gabrb1 and Gabrg1 receptors.