

**STOmics**

**Stereo-seq**  
**ANALYSIS WORKFLOW**  
**SOFTWARE SUITE**  
**USER MANUAL**

Software Version: V7.1

Manual Version: A9.0

# REVISION HISTORY

**Manual Version:** A0  
**Software Version:** V1.0.0  
**Date:** Nov. 2021  
**Description:** Initial release

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**Manual Version:** A1  
**Software Version:** V2.1.0  
**Date:** Dec. 2021  
**Description:**

- **New feature:** addition of manual registration function, some fine-tuning will be automatically performed after manually registering;
  - **Improvement:** performance improvement for **mapping**; the order of sequencing saturation calculation has switched, in v2.1.0 we only compute the saturation of tissue-covered region;
  - **Bug fix:** fixed the bug of indexing at **mapping** step; fix the bug of long waiting time at **register** step.
- 

**Manual Version:** A1.1  
**Software Version:** V2.1.0  
**Date:** Jan. 2022  
**Description:**

- Add error handling;
  - Update demo output.
- 

**Manual Version:** A2  
**Software Version:** V4.1.0  
**Date:** Apr. 2022  
**Description:**

- **New feature:** support to process fused microscopic images; employ Stereopy in clustering; new gene expression matrix file format; including a file format convertor and a **mapping** memory estimator;
- **Improvement:** performance improvement for **mapping**; update gene annotation approach in **count**; update image stitching and tissue segmentation model for better performance on image stitching and segmentation for tissue with voids.

**Manual Version:** A3  
**Software Version:** V5.1.3  
**Date:** Aug. 2022  
**Description:**

- **New feature:** addition of cell segmentation on microscopic image in **register** pipeline module and **cellCut** pipeline module to extract cell expression matrix; design IPR file to store image processing record information and TIFF images produced in **register**; output exon expression matrix along with total MID count; addition of cell clustering analysis; addition of cell bin statistic result and image information in HTML report; add pipeline modules to support single-end FASTQ data; addition of option for selecting multi-mapped reads; addition of score system in image processing;
  - **Improvement:** addition of poly A filtration in **mapping**; performance improvement for **merge**, **tissueCut** and **saturation**; add data scaling and upgrade clustering analysis pipeline;
  - **Bug fix:** fix the bug of plotting scatter plot in **tissueCut**, review and modify the ambiguous metrics names and explanations in HTML report.
- 

**Manual Version:** A4  
**Software Version:** V5.4.0  
**Date:** Nov. 2022  
**Description:**

- **New feature:** render a more elegant way of organizing SE FASTQs input into **mapping**; addition of header for **count** output TXT file; upgrade **ipr2img** to **imageTools** which allows you to merge TIFF images to check segmentation result and plot templates on the panoramic image or registered image to check the result of stitching and registration;
- **Improvement:** change data struct of gene index in the GEF file from uint16 to uint32 to store more genes;
- **Bug fix:** fix the typo in the manual.

Manual Version: A3.1  
 Software Version: V5.1.3  
 Date: Dec. 2022  
 Description:

- **Bug fix:** fix the typo in the manual.
- 

Manual Version: A5  
 Software Version: V5.5.0 - V5.5.2  
 Date: Jan. 2023  
 Description:

- **New feature:** addition of `manualRegister` and `lasso` pipeline modules, which acquire parameters and essential files from offline visualization software **StereoMap**; addition of error code function in each module, and the explanation has been organized in appendix D;
  - **Improvement:** upgrade `tissueCut` pipeline module for better performance and improved accuracy of tissue recognition directly from gene expression matrix; upgrade clustering analysis pipelines; addition of image layers in HTML report which allow users to switch images displayed with gene expression distribution plot; the stitching deviation heatmap(s) and the frame of axes in the HTML report have been adjusted according to the registration parameters.
- 

Manual Version: A5  
 Software Version: V5.5.3  
 Date: Mar. 2023  
 Description:

- **Improvement:** upgrade `mapping` pipeline module, which performs polyA filtering after CID mapping, for improved statistical output in `report`.

Manual Version: A6  
 Software Version: V6.0  
 Date: Mar. 2023  
 Description:

- **New feature:** addition of `rRNAremove` switch in `mapping`, off by default, to count and filter rRNA reads using the reference genome with the addition of rRNA information. rRNA count and ratio are recorded in the output file; support the scenario with the combination of DAPI and mIF (multiple immunofluorescence images), correspondingly generating registered, tissue segmentation, and cell segmentation results; support continuously processing image files output from each module of ImageStudio;
  - **Improvement:** reconstructed `tissueCut` module pipeline, with the addition of parameter `-t` which means the number of used CPUs, to greatly improve the calculation efficiency; modified parameter `-g` in `img2rpi` module to a two-layer structure, upgraded RPI file format to support storing and organizing multiple stained microscopy images in groups; displayed pseudo colors (up to 7 colors) on the bottom layer of clustering results in HTML report, when handling the scenario of mIF in `report` module;
  - **Bug fix:** fix the bug caused by version compatibility when generating CGEF file.
- 

Manual Version: A6.1  
 Software Version: V6.0.1 & V5.4.4  
 Date: Apr. 2023  
 Description:

- **Bug fix:** fix the bug that the execution path of `checkGTF` function was invalid.

**Manual Version:** A6.1  
**Software Version:** V6.0.2  
**Date:** Jun. 2023  
**Description:**

- **Improvement:** removed the dependence of parameters related to stitched-image stitching-evaluation from different versions of QC. The evaluation of stitched-image stitching is completed by register module; prompt an error when <StainType> in .ipr file is '-'; supported Zeiss microscope-stitched image;
- **Bug fix:** for register, fixed the issue that the module was unable to get .czi file path; fixed the issue of doing cell segmentation in rapidRegister for manually-stitched data; fixed the issue that the registered .ipr file that could not be registered again; expanded the chip-prefix list and fixed the track line template acquisition error. For imageTools, fixed the issue that bin\_100 of cellMask in the SN.rpi file did not save corresponding metadata; fixed the issue that the merge could not be performed on images with the same width and height.

---

**Manual Version:** A7  
**Software Version:** V6.1  
**Date:** Jul. 2023  
**Description:**

- **New feature:** supported outputs of Valid CID Reads and un-mapped reads in FASTQ format; supported QC-failed but manually processed images as inputs for subsequent workflow; addition of tissue area (nm<sup>2</sup>) in corresponding GEF file; supported generating GEF file of labeled region; addition of quality control alerts and tissue segmentation display in report;
- **Improvement:** updated mapping module to improve computational efficiency; updated the error alerts on checking the length of the gene name, in checkGTF and count modules.

**Manual Version:** A7  
**Software Version:** V6.1.1  
**Date:** Aug. 2023  
**Description:**

- **Bug fix:** for manualRegister, fixed the issue that obtaining information from manual registration file .json incorrectly resulted in wrong width and height of images; for tissueCut, corrected the statistical method of Reads\_under\_tissue item in tissueCut, which now represents "Number of reads with position prior to filtration under tissue coverage"; for report, compatible with the .ipr file which is failed with image quality control (QC) and has no record of heat map matrix information needed for html report.

---

**Manual Version:** A7  
**Software Version:** V6.1.2  
**Date:** Sep. 2023  
**Description:**

- **Bug fix:** for manualRegister, fixed the offset calculation error of which the scale direction was incorrect.

---

**Manual Version:** A7  
**Software Version:** V6.1.2  
**Date:** Oct. 2023  
**Description:**

- **Bug fix:** for imageTool, fixed the problem that manually registered images were incorrectly cropped after scale operation; for report, fixed the missing percentage value of floating text box for 'Sequencing Saturation', in HTML diagram.



**Manual Version:** A8  
**Software Version:** V7.0  
**Date:** Nov. 2023

**Description:**

- **New feature:** supported H&E image in pipeline; addition of `cellCorrect` module for expanding cell boundaries; addition of `cellChunk` module for displaying cell expression heatmap in StereoMap; addition of new parameter in `spatialCluster` for adjusting Leiden clustering resolution; addition of output GEF file in `lasso` module;
  - **Improvement:** performance improvement in `count` and `register` modules; updated tissue and cell segmentation model for nuclei-stain image; supported for using GPU in `register` module to accelerate computing.
- 

**Manual Version:** A9  
**Software Version:** V7.1  
**Date:** Mar. 2024

**Description:**

- **New feature:** addition of Proteomics & Transcriptomics jointly analysis required modules, including `mapping-SP`, `calibration`, `spatialCluster-SP`, `cellCluster-SP`, `multiomicsAnalysis` and `report-PT`; supported Proteomics mode in modules: `tissueCut` and `cellCut`; addition of a new small tools: `MIDFilter`, which supported extract GEF based on MID range.

**Note: Please download the latest version of the manual and use it with the software specific to this manual.**

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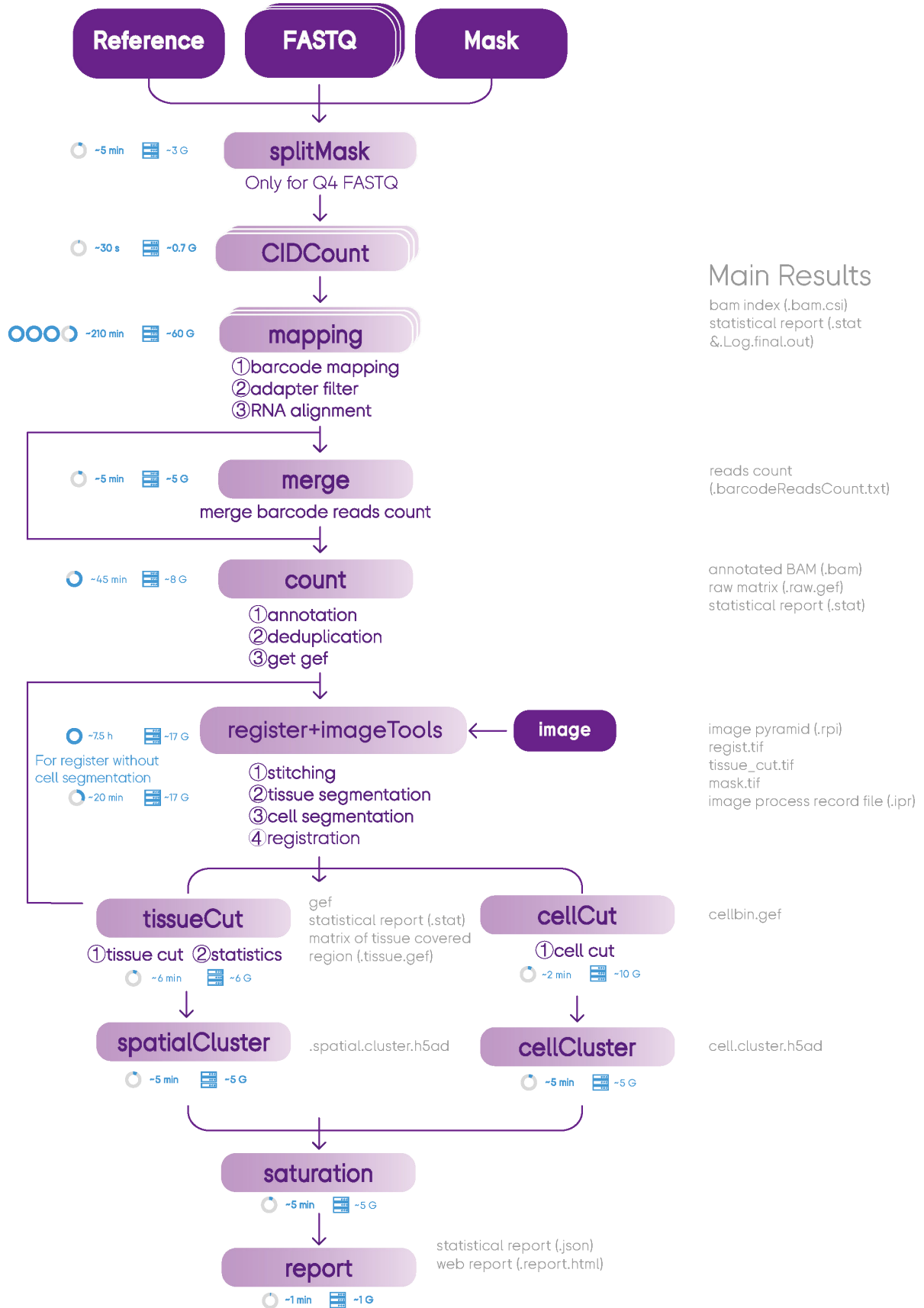
# WORKFLOW

📁 1 G reads

64 bit CentOS/RedHat 7.8  
64 bit Ubuntu 20.04

Minimum requirements: 8 cores 128 G 1 TB

Higher requirements: 24 cores 256 G 1 TB



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**CRITICAL STEPS:** Pay extra attention to these instructions/steps to avoid problematic results.



**SOLUTION:** Provides a solution to handling common errors.

# CHAPTER 1

## Stereo-seq ANALYSIS WORKFLOW SOFTWARE SUITE

## 1.1. Software Introduction

Stereo-seq Analysis Workflow<sup>1</sup> (SAW) software suite is a set of pipelines bundled to position sequenced reads to their spatial location on the tissue section, quantify spatial expression and visually present spatial expression distribution. SAW processes the sequencing data of Stereo-seq<sup>2</sup> to generate spatial expression matrices, and then users could take these files as the starting point to perform downstream analysis. SAW includes thirteen essential and suggested pipelines and auxiliary tools for supporting other handy functions:

- **splitMask:** Split Stereo-seq Chip T mask file into several pieces according to CID indexing in the Q4 FASTQ files.
- **CIDCount:** Count CIDs in the Stereo-seq Chip T mask file and roughly estimate memory required to do mapping.
- **mapping:** Correspond *in situ* captured sequenced reads recorded in FASTQ<sup>3,4</sup> files by Stereo-seq with their spatial information. It also aligns reads to the reference genome and generates coordination sorted BAM files.
- **merge (optional):** Combine CID (same as barcodes) listed files with reads count from multiple runs of mapping. Only for an analysis that requires to combine multiple pairs of FASTQ.
- **count:** Read BAM files generated from mapping to perform gene annotation, de-duplication, and gene expression analysis on the aligned reads.
- **register:** Align microscopic tissue staining image with expression matrix file (GEF) generated from count. register is an optional pipeline when image fails QC or input image is absent.
- **imageTools:** Convert TIFF images from IPR, such as template-aligned stitched TIFF image, binarized tissue segmentation and cell segmentation images. Optional module when image fails QC or input image is absent.
- **tissueCut:** Identify tissue coverage area on the chip and extract gene expression matrix of the corresponding spatial location by taking inputs from both count and register or count pipeline alone. If you do Proteomics & Transcriptomics jointly analysis, the calibrated expression matrices are from calibration.
- **spatialCluster & spatialCluster-SP:** Perform clustering analysis for spots (bin200) according to the expression matrices of the tissue coverage area generated from tissueCut.
- **cellCut:** Identify cell nuclei area on the staining image and extract expression matrices of the corresponding spatial location by taking inputs from both count and register&imageTools pipeline. If you do Proteomics & Transcriptomics jointly analysis, the calibrated expression matrices are from calibration. Optional module when image fails QC or input image is absent.
- **cellCorrect:** Adjust cell coverage region based on the aligned cell nuclei segmentation image generated from register and imageTools. Then, output expression matrix of the adjusted cells in cell bin GEF and GEM formats.
- **cellCluster & cellCluster-SP:** Perform clustering analysis for cell bins according to the expression matrices which are generated from cellCorrect. Optional module when image failed QC or input image is absent.
- **saturation:** Calculate sequencing saturation of tissue coverage area based on the file that was used for sampling data generated from count or mapping-SP.
- **report:** Generate a JSON format statistical summary report that integrates the analysis result from each step, as well as an HTML web analysis report, and shows spatial expression distribution of genes, key statistical metrics, sequencing saturation plots, clustering analysis results. Depending on the image input state and register mode, HTML reports may or may not have cell bin statistical data and image processing key results.
- **mapping-SP:** Correspond *in situ* captured sequenced reads recorded in FASTQ<sup>3,4</sup> spatial information. It aligns reads to the customised protein database and generates GEF files for protein.
- **calibration:** Read two GEF files generated from count and mapping-SP to perform spatial coordinate alignment.





- **multiomicsAnalysis:** Integrate RNA and protein data and compute the latent space by Total Variational Inference<sup>14</sup>. Perform clustering analysis for latent space and do one-vs-all differential expression analysis to find marker genes and proteins.
- **report-PT (Proteomics & Transcriptomics):** Generate a JSON format statistical summary report that integrates the analysis result from each step, as well as an HTML web analysis report folder, and shows spatial expression distribution of genes & proteins, key statistical metrics, sequencing saturation plots, clustering analysis results, protein proportion and multiomics analysis. Depending on the image input state and register mode, HTML reports may or may not have cell bin statistical data and image processing key results.

Other handy functions:

- **Other applications of cellCut:** Manipulate GEF file.
- **checkGTF:** Check GTF/GFF file is prepared in the correct format, otherwise, re-format one that meets the compatibility requirements for count.
- **Other applications of imageTools:** 1) Merge two to three TIFF images in R-G-B order, which is useful for checking segmentation result. 2) Plot templates on the panoramic image to assist in the evaluation of stitching and registration result. 3) Write TIFF images into RPI format.
- **manualRegister:** Acquire manual registration operation parameters from **StereoMap visualization software** and run `manualRegister` to modify registration records in IPR. Users can turn on the "fine-tune" switch to let `manualRegister` perform an automatic adjustment to the manually operated result to make the registration more precise.
- **lasso:** Acquire one or multiple cell or spatial expression matrix subsets according to the GEOJSON file, which stores the coordinates data of manually delineated region(s) from **StereoMap visualization software**.
- **cellChunk:** Generate encoding pre-computed data used for **StereoMap** rendering.
- **MIDFilter:** Perform manually filter spatial expression matrixes by MID range acquired from **StereoMap**.

## 1.2. System Requirements

SAW runs on Linux systems that meet the following minimum requirements:

|   |
|---|
| 8-core Intel or AMD processor (>24 cores recommended) |
| 128GB RAM (>256GB recommended)                        |
| 1TB free disk space or higher                         |
| 64-bit CentOS/RedHat 7.8 or Ubuntu 20.04              |

To install and run SAW, please install one kind of the following softwares:

|   |
|---|
| <b>docker</b> <sup>5</sup> : version 20.10.8 or higher  |
| <b>singularity</b> <sup>6</sup> : version 3.8 or higher |

## 1.3. Related Software

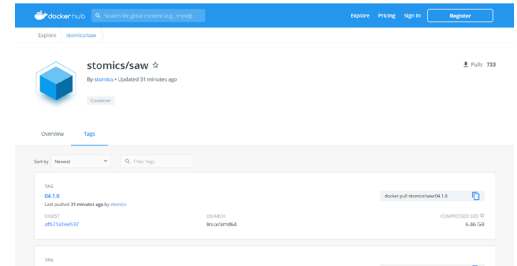
- **ImageStudio:** **ImageStudio** is a fully upgraded image process desktop application. It contains four main image processing modules: image QC, manual stitching, manual tissue segmentation, and manual cell segmentation. The outputs of each module can be input into SAW for further analysis. SAW v7.1 recommends **ImageStudio** version  $\geq$  v3.0.
- **StereoMap:** **StereoMap** is an HD visualization desktop application intended for displaying Stereo-seq analysis results. SAW outputs such as gene expression matrix GEF file, image RPI and IPR files, clustering results can be visualized via **StereoMap**. SAW v7.1 recommends **StereoMap** version  $\geq$  v3.1.

## 1.4. SAW Docker Image Installation

SAW is delivered as a docker image that bundles all of its required software dependencies. You can pull the SAW docker image from Docker Hub to your local system and run analyses offline.

Please download the latest version of the software and use it with the corresponding [Stereo-seq Analysis Workflow Software Suite User Manual](#) version.

Docker Hub link: <https://hub.docker.com/r/stomics/saw/tags>



We support using Singularity and Docker to install and run the SAW.

Here we take **SAW version 6.1** as an example.

### 1.4.1. SAW Installation via Singularity



Please replace the yellow highlighted inputs with your own data path.

Step 1: Pull the SAW docker image (2 options):

```
$ singularity build SAW_v7.1.sif docker://stomics/saw:07.1.0 ## option 1
$ singularity build --sandbox SAW_v7.1/ docker://stomics/saw:07.1.0 ## option 2
```

For non-root users, especially who don't have enough space in the `/home/` directory, please try:

```
$ export SINGULARITY_CACHEDIR=/path/to/build
$ singularity build --sandbox SAW_v7.1/ docker://stomics/saw:07.1.0
```

Step 2: Run pipelines (3 options):



**Please Note!** All the requested paths need to be mounted before input. For example, it is necessary to bind directories that store input files (`/path/to/data`), reference genome (`/path/to/genomeDir`), and outputs (`/path/to/output`).

```
$ export SINGULARITY_BIND="/path/to/data,/path/to/genomeDir,/path/to/output"
```

Option 1: Run pipelines within the container from the host system.

```
$ /path/to/SAW_v7.1.sif <application> ## option 1.1
$ singularity exec /path/to/SAW_v7.1.sif <application> ## option 1.2
```

Option 2: Shell into the SAW container and interactively run bash commands. Run `exit` to exit the environment.

```

$ /path/to/SAW_v7.1.sif /bin/bash ## option 2.1
Singularity>
Singularity> <shell-command>
Singularity> exit
$
$ singularity shell /path/to/SAW_v7.1.sif ## option
2.2 Singularity>
Singularity> <shell-command>
Singularity> exit
$
$ singularity shell SAW_v7.1 ## option 2.3 for sandbox
Singularity>
Singularity> <shell-command>
Singularity> exit
$

```

Option 3: Use “`-B directory on the host-machine:directory in the container`” to mount a host directory into the container and execute the command in the container.

```

$ singularity shell -B /path/to/directory/on/the/host-machine:/path/to/directory/
mounted/in/the/container /path/to/SAW_v7.1.sif
Singularity>
Singularity> <shell-command>
Singularity> exit
$

```

\* Please be noted that these two lines belong to the same line of command.

## 1.4.2. SAW Installation via Docker

Step 1: Pull the SAW docker image

```
$ docker pull stomics/saw:07.1.0
```

Step 2: Run pipelines

```
$ docker run -d -v /path/to/data:stomics/saw:07.1.0 /bin/sh -c "<shell-command>"
```

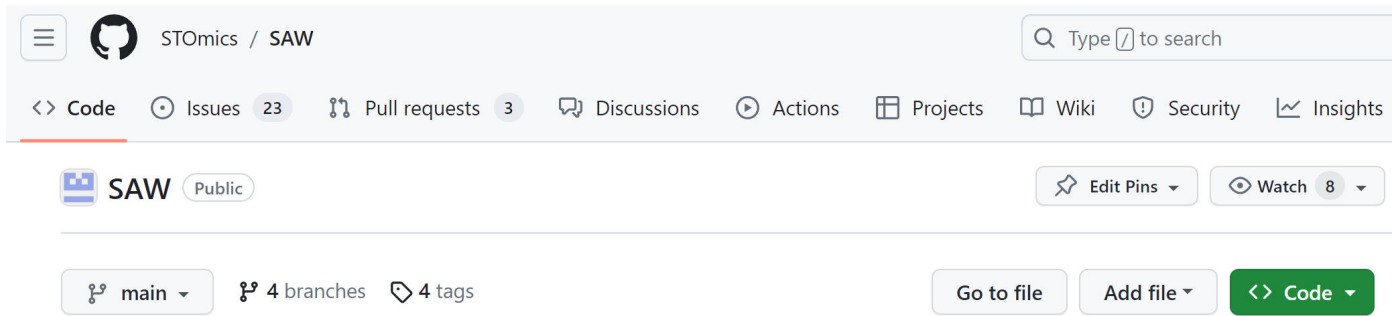
## 1.5. SAW Github

SAW GitHub: <https://github.com/STOmicS/SAW>

Please visit GitHub for instruction regarding the **installation of singularity** and **indexing reference genome**. The page also provides **SAW shell script** examples for users.



**Please Note! Please build your reference before running SAW analysis.**



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## 1.6. SAW Test Data

SAW test data can be downloaded from SAW GitHub page. Key outputs are shown in [Chapter 3 Test Data Demonstration](#). The reference genome version for SAW testing is:

- genome-build: GRCm38.p6
- genome-version: GRCm38
- genome-date: 2012-01
- genome-build-accession: NCBI:GCA\_000001635.8

## 1.7. SAW Output File Format

Please check [Stereo-seq File Format Manual](#) to get more information on SAW output files format.

# CHAPTER 2

## SAW PIPELINES & ARGUMENTS

## 2.1 splitMask

`splitMask` is designed for splitting Stereo-seq Chip T mask file according to the SE FASTQ CID. The split count is directly related to the number of split FASTQs while writing FASTQ out from sequencer.

Running `splitMask` requires the following files:

- Stereo-seq Chip T mask file (**.h5**)
- 🕒 Expected running time for 1 cm x 1 cm chip: ~5 min, Memory: ~3G

⋯ As input sequencing data, both Q40 FASTQ and Q4 FASTQ from Stereo-seq, which store the original sequencing data, are supported in SAW pipelines. Differences between them are mainly reflected in file size and how data and information are written in. Q40 FASTQ, also called PE FASTQ, has a pair of read files, read 1 and read 2. Q4 FASTQ, also called SE FASTQ, is an output format with only one file, but saves file storage space. More details in [Stereo-seq File Format Manual](#).

An example of Q4 FASTQ file path usually displayed as below:



### 2.1.1 Arguments and Options

Table 2-1 `splitMask` Arguments and Options

| Parameter index | Function  |
|-----------------|---|
| [1]             | (Required) Stereo-seq Chip T mask file path (.h5).  |
| [2]             | (Required) Output directory stores split mask files.  |
| [3]             | (Required) Set the number of threads to be used.  |
| [4]             | (Optional) Split count. This count number needs to be set the same as Q4 FASTQ count. The options are the powers of 4, and the commonly used options are 16 and 64. If a library contains two barcodes sequenced in the same lane and the two barcodes were split when written out FASTQs, then the split count is 16; otherwise, if the barcodes were not split, then 64.  |
| [5]             | (Optional) CID position, commonly used option is “2_25”. <code>splitMask</code> uses 24 bases (out of 25, 25 is the read length of CID) to split Stereo-seq Chip T mask file. This CID position parameter is a string that combines two index numbers with “_”. The two numbers indicate the start and the end base index in the CID, and the bases in this range are used for splitting. For example, “2_25” means starting from the 2 <sup>nd</sup> base to the 25 <sup>th</sup> base used for splitting mask file. |



## 2.1.2 Usage Example



Please Note! Replace **{SN}** with your Stereo-seq Chip T serial number (SN, e.g. SS200000135TL\_D1 or A00135D1)

```
$ mkdir /path/to/output/00.splitmask
$ singularity exec SAW_v7.1.sif splitMask \
  /path/to/data/{SN}.barcodeToPos.h5 \ ## Mask path
  /path/to/output/00.splitmask \ ## output directory
  8 \ ## threads
  16 \ ## split count
  2_25 ## CID position
```

## 2.1.3 Outputs

The output files of **splitMask** are organized as below. The indices of the output files are corresponding to the split list index of the Q4 FASTQ files.

```
$ tree /path/to/output/00.splitmask
/path/to/output/00.splitmask
|-- 01.SN.barcodeToPos.bin
|-- 02.SN.barcodeToPos.bin
|-- 03.SN.barcodeToPos.bin
|-- 04.SN.barcodeToPos.bin
|-- 05.SN.barcodeToPos.bin
|-- 06.SN.barcodeToPos.bin
|-- 07.SN.barcodeToPos.bin
|-- 08.SN.barcodeToPos.bin
|-- 09.SN.barcodeToPos.bin
|-- 10.SN.barcodeToPos.bin
|-- 11.SN.barcodeToPos.bin
|-- 12.SN.barcodeToPos.bin
|-- 13.SN.barcodeToPos.bin
|-- 14.SN.barcodeToPos.bin
|-- 15.SN.barcodeToPos.bin
`-- 16.SN.barcodeToPos.bin

0 directories, 16 files
```

## 2.2 CIDCount

**CIDCount** is a program for computing the number of CIDs in the Stereo-seq Chip T mask file and roughly estimating memory used in **mapping**.

Running **CIDCount** requires the following files:

- Stereo-seq Chip T mask file (**.h5**)
- 🕒 Expected running time for 1 cm x 1 cm chip: ~30 s, Memory: ~0.7G





## 2.2.1 Arguments and Options

Table 2-2 CIDCount Arguments and Options

| Parameter | Function   |
|-----------|--|
| <b>-i</b> | (Required) Stereo-seq Chip T mask file path (.h5 or .bin). |
| <b>-s</b> | (Optional) A string of species name.                       |
| <b>-g</b> | (Required) Genome file size in GB.                         |

## 2.2.2 Usage Example

For Q40 FASTQ input cases, run **CIDCount** as below:

```
$ singularity exec SAW_v7.1.sif CIDCount \
  -i /path/to/data/{SN}.barcodeToPos.h5 \ ## Stereo-seq Chip T mask file path
  -g {genomeSize} ## genome file size in GB, can be acquired by "ls -l
  --block-size=GB ${Genome file of the species after STAR indexing}"
```

For Q4 FASTQ input cases that require to run **splitMask** first, users may run **CIDCount** only once because the results for each individual small mask are close to each other. For chip size larger than 1 cm x 1 cm, we would recommend to run **CIDCount** for each CID class/small mask.



**Please Note!** **{index}** needs to be replaced by the real index number of the split mask file.

```
$ singularity exec SAW_v7.1.sif CIDCount \
  -i /path/to/output/00.splitmask/{index}.{SN}.barcodeToPos.bin \ ## Ste-
  reo-seq Chip T mask file path
  -g {genomeSize} ## genome file size in GB, can be acquired by "ls -l
  --block-size=GB ${Genome file of the species after STAR indexing}"
```

## 2.2.3 Outputs

The output of **CIDCount** is shown as below,

```
$ singularity exec SAW_v7.1.sif CIDCount -i SN.barcodeToPos.h5 -s mouse -g 3
645784920 ## CID count
62 ## estimated memory for mapping
```


## 2.3 mapping

Each Stereo-seq sequenced read contains a CID sequence which is used as a key to spatially map the read back to its original location on the tissue section. **mapping** pipeline matches CID of the original sequencing reads stored in the FASTQ file with the records of CID-coordinates key-value pairs saved in the Stereo-seq Chip T mask file (allow 1 mismatch). Coordinate information for reads that CID could be paired with, based on the records of the mask file, will be added. Coordinate information for reads that CID could be paired with will be added based on the records of the mask file. Reads that get the coordinate annotations are Valid CID mRNA Reads (**Valid CID Reads**). After discarding reads with unqualified MID reads which do not satisfy further analysis, filtering reads with adapter, and filtering short reads (read length less than 30 after trimming consecutive A bases), the filtered **Valid CID Reads** are the **Clean Reads**. **mapping** pipeline maps **Clean Reads** to the reference genome, and output sorted BAM<sup>7</sup> format alignments and summary report.

If concerned about rRNA count and filtration, necessary modification to the reference genome should be completed beforehand, mainly including 2 steps: 1) add rRNA information into the FASTA file, with the suffix '\_rRNA' on the chromosome column, like '1\_rRNA', to distinguish them from original reference; 2) build index using modified reference genome.

Running **mapping** requires the following files:

- Stereo-seq sequenced reads FASTQ files (**.fq.gz**)
- Stereo-seq Chip T mask file (**.h5**)
- Indexed reference genome
- bcPara file (**.bcPara**), please check the content of **Table 2-4**

 Expected running time for ~1G reads: ~4 h, Memory: ~67G



**Notice to rebuild reference index, with the same command input as before. From SAW V6.1 onwards, SAW reconstructed the construction of reference index and mapping algorithms for more efficient computational performance and reduced time cost.**

### 2.3.1 Arguments and Options

As **mapping** encapsulate STAR<sup>8</sup> function, it accepts additional options beyond those shown in the table below.

Table 2-3 **mapping** Arguments and Options

| Parameter                                  | Function   |
|--|--|
| <b>--outSAMattributes spatial</b>          | Set to turn on spatial BAM file format mode.   |
| <b>--outSAMtype BAM SortedByCoordinate</b> | (STAR option) Set output BAM file sorted by coordinate.  |
| <b>--genomeDir</b>                         | (STAR option) Path to the directory where the genome indices are stored.                                 |
| <b>--runThreadN</b>                        | (STAR option; defaults to 1) Set the number of threads to be used. Usually set to 8 or higher.           |
| <b>--outFileNamePrefix</b>                 | (STAR option) Custom output file prefix.   |
| <b>--sysShell /bin/bash</b>                | (STAR option) Path to the shell binary.  |
| <b>--stParaFile</b>                        | (Required) Create a parameter file that defines CID mapping options. Options are specified in Table 2-4. |
| <b>--readNameSeparator \"</b><br><b>\"</b> | (STAR option) Character(s) separating the part of the read names that will be trimmed in output.         |

| Parameter                          | Function  |
|------------------------------------|---|
| <code>--limitBAMsortRAM</code>     | (STAR option) Maximum available RAM (bytes) for sorting BAM.  |
| <code>--limitOutSJcollapsed</code> | (STAR option) Max number of collapsed junctions.  |
| <code>--limitIObufferSize</code>   | (STAR option) Max available buffers size (bytes) for input/output, per thread.  |
| <code>--outSAMmultNmax</code>      | (STAR option; defaults to -1) Max number of alignments that will be written in output BAM file for a read. Recommend setting to 1 to reduce disk space storage. |

Table 2-4 `mapping --stParaFile` Arguments and Options

| Parameter                        | Function  |
|----------------------------------|---|
| <code>in</code>                  | (Required) Path to the Stereo-seq Chip T mask file (PE) or split mask file (SE).  |
| <code>in1</code>                 | (Required) Path to the FASTQ file. For Q40 sequences specify the path to the FASTQ file of read1 here. For Q4 sequences, input FASTQ file with the same split index as the mask file. A more elegant way to input a long list of Q4 FASTQs is to organize them in a <code>FQ_{index}.list</code> file. Please check <a href="#">2.3.2 Usage Example Q4 scenario 2</a> to get more information.  |
| <code>in2</code>                 | (Optional) Path to the FASTQ file of read2. Only valid for Q40 sequencing.  |
| <code>barcodeReadsCount</code>   | (Required) Mapped CID list file with read counts for each CID. This is an output file in <code>mapping</code>   |
| <code>barcodeStart</code>        | (Required; defaults to 0) CID start position. Set to 0.   |
| <code>barcodeLen</code>          | (Required; defaults to 25) CID length. Set to 25 for Q40, 24 for Q4.  |
| <code>umiStart</code>            | (Required; defaults to 25) MID start position.  |
| <code>umiLen</code>              | (Required; defaults to 10) MID length.  |
| <code>mismatch</code>            | (Required; defaults to 0) Max mismatch tolerant. Usually set to 1 in <code>mapping</code> .   |
| <code>bcNum</code>               | (Required) CID count in mask. Please check <a href="#">2.2 CIDCount</a> for more information.   |
| <code>polyAnum</code>            | (Optional) Number of consecutive A in the read that will be trimmed. Recommend to set this value to 15.   |
| <code>mismatchInPolyA</code>     | (Optional) Number of mismatch bases in searching poly A. Recommend to set this value to 2   |
| <code>rRNAremove</code>          | (Optional; default to disable) Remove rRNA reads. Note that the precondition of the switch is the reference genome combined with rRNA information. If not, this filtering function will not work anyway. Using the reference genome with the addition of rRNA, reads related to rRNA are not filtered by default, namely the switch is off. And the number and the ratio of rRNA reads in the statistical output file are both 0. When the switch is on, as long as the query reads are mapped to rRNA reference, they will be removed. rRNA count and ratio are recorded in the output file. |
| <code>validCidFq</code>          | (Optional; default to disable) Output <b>Valid CID Reads</b> in FASTQ format after CID mapping. Similar to Q4 FASTQ format but with different first row of each read, for instance, "@V350044321L1C001R0020993658 Cx:i:10413 Cy:i:7737 D3450E0D391E EC7FF", where Cx and Cy represent the decoded coordinates and are added after readID.   |
| <code>outUnMappedFq</code>       | (Optional; default to disable) Output un-mapped reads in FASTQ format after mapping <b>Clean Reads</b> to the reference genome. Similar to Q4 FASTQ format but with different first row of each read, for instance, "@V350044321L1C001R0020993658 Cx-i:10413 Cy:i:7737 D3450E0D391E EC7FF", where Cx and Cy represent the decoded coordinates and are added after readID.   |
| <code>bcMappingRateCutoff</code> | (Optional; default to disable) Set the minimum CID mapping rate, like 'bcMappingRateCutoff=0.1'. If the actual CID mapping rate less than this ratio, abort <code>mapping</code> .  |

## 2.3.2 Usage Example

Two scenarios for preparing `mapping --stParaFile` input file `{lane}.bcPara` with Q40 FASTQ input:



Please Note! Replace `{SN}` with your Stereo-seq Chip T serial number (SN, e.g. SS200000135TL\_D1 or A00135D1), and `{lane}` with the FASTQ lane name prefix (e.g. E100026571\_L01)

The same applies to all examples.

Q40 scenario 1: Prepare `{lane}.bcPara` file for Q40 one pair FASTQ as `mapping` input:

```
$ mkdir /path/to/output/01.mapping
$ vim /path/to/output/01.mapping/{lane}.bcPara
in=/path/to/data/{SN}.barcodeToPos.h5
in1=/path/to/data/{lane}_read_1.fq.gz
in2=/path/to/data/{lane}_read_2.fq.gz
barcodeReadsCount=/path/to/output/01.mapping/{lane}.barcodeReadsCount.txt
barcodeStart=0
barcodeLen=25
umiStart=25
umiLen=10
mismatch=1
bcNum=645784920 ## Input the first line from output of CIDCount
polyAnum=15
mismatchInPolyA=2
```

Q40 scenario 2: Prepare `{lane}.bcPara` file for Q40 multiple pair of FASTQ as `mapping` input:

```
...
$ mkdir /path/to/multi_lane_output/01.mapping
$ vim /path/to/multi_lane_output/01.mapping/{lane}.bcPara
in=/path/to/data/{SN}.barcodeToPos.h5
in1=/path/to/data/{lane}_read_1.fq.gz
in2=/path/to/data/{lane}_read_2.fq.gz
barcodeReadsCount=/path/to/multi_lane_output/01.mapping/{lane}.barcodeReadsCount.txt
barcodeStart=0
barcodeLen=25
umiStart=25
umiLen=10
mismatch=1
bcNum=645784920 ## Input the first line from output of CIDCount
polyAnum=15
mismatchInPolyA=2
```

Run **mapping** for Q40 input:

```

$ singularity exec SAW_v7.1.sif mapping \
  --outSAMattributes spatial \
  --outSAMtype BAM SortedByCoordinate \
  --genomeDir /path/to/genomeDir \
  --runThreadN 8 \
  --outFileNamePrefix /path/to/output/01.mapping/{lane}. \
  --sysShell /bin/bash \
  --stParaFile /path/to/output/01.mapping/{lane}.bcPara \
  --readNameSeparator "\" \" \
  --limitBAMsortRAM 38582880124 \
  --limitOutSJcollapsed 10000000 \
  --limitIObufferSize=280000000 \
  --outBAMsortingBinsN 50 \
  > /path/to/output/01.mapping/{lane}.run.log

```

Two scenarios for preparing **mapping** `--stParaFile` input file `{index}.bcPara` with Q4 FASTQ input:

**Q4 scenario 1:** Prepare `{index}.bcPara` file for Q4 FASTQ that contains one barcode in library or did not split barcode when writing FASTQ:



**Please Note!** `{index}` need to be replaced by the index of the split mask file which is corresponding to the index of the Q4 FASTQ file.

```

$ mkdir /path/to/output/01.mapping
$ vim /path/to/output/01.mapping/{index}.bcPara
in=/path/to/output/00.splitmask/{index}.{SN}.barcodeToPos.bin ## split mask file
in1=/path/to/data/{lane}_{index}.fq.gz ## {index}th FASTQ file in {lane}
barcodeReadsCount=/path/to/output/01.mapping/{index}.barcodeReadsCount.txt
barcodeStart=0
barcodeLen=24
umiStart=25
umiLen=10
mismatch=1
bcNum=38284877 ## Input the first line from output of CIDCount
polyAnum=15
mismatchInPolyA=2

```

**Q4 scenario 2:** Prepare `{idx}.bcPara` file for Q4 FASTQ that has multiple barcodes in the library and split when writing FASTQ:



**Please Note!** `{index}` needs to be replaced by the real index number of the split mask file and `{idx}` needs to be replaced by the index number of the input FASTQ file. For example, `{index}` and `{idx}` of the first barcode group are "01-16" and "01-16", `{index}` and `{idx}` of the second barcode group are "01-16" and "17-32".

```

$ mkdir /path/to/output/01.mapping
$ vim /path/to/output/01.mapping/{idx}.bcPara
in=/path/to/output/00.splitmask/{index}.{SN}.barcodeToPos.bin ## split mask file
in1=/path/to/data/{lane}_{index}.fq.gz ## {index}th FASTQ file in {lane}. FASTQ
files for different barcode usually stores in different directory, so /path/to/
data/ might change to /path/to/data/{barcode_n}
barcodeReadsCount=/path/to/ouptut/01.mapping/{idx}.barcodeReadsCount.txt
barcodeStart=0
barcodeLen=24
umiStart=25
umiLen=10
mismatch=1
bcNum=38284877 ## Input the first line from output of CIDCount
polyAnum=15
mismatchInPolyA=2

```

In case there are too many FASTQ files that need to be processed, an easier way is to organize them into a `FQ_{index}.list` file. The requirement for preparing a `FQ_{index}.list` file is to gather all the FASTQs with the same index as the split mask together, since these files are all split by the same logic.

Q4 FASTQ name

`/path/to/data/E100026571_L01/barcode_2/E100026571_L01_2_16.fq.gz`

Get the list input conveniently like:

```

$ sh Get_FASTQ_list.sh <FASTQDirList> <outdir> <SN>

FASTQDirList = <Q4 FASTQ directory list which record directory paths line byline>
outdir = <directory to output FQ.list files>
SN = <Stereo-seq chip serial number>

```

[https://github.com/STOmics/SAW/blob/main/Scripts/Get\\_FASTQ\\_list.sh](https://github.com/STOmics/SAW/blob/main/Scripts/Get_FASTQ_list.sh)

An example to display `FQ.list` file :

```

$ cat SN_Q4_fastq_16.list ## a FASTQ list file gathers all the FASTQs whose index
is 16
/path/to/data/lane_1_16.fq.gz
/path/to/data/lane_2_16.fq.gz

```

Then input the path of `FQ_{index}.list` file for `in1` parameter in the `{index}.bcPara` file. The `{index}` of `FQ_{index}.list` and the `{index}` of split mask file have to be the same.

```

$ mkdir /path/to/output/01.mapping
$ vim /path/to/output/01.mapping/{index}.bcPara
in=/path/to/output/00.splitmask/{index}.{SN}.barcodeToPos.bin ## split mask file
in1=/path/to/output/{SN}_Q4_fastq_{index}.list
barcodeReadsCount=/path/to/output/01.mapping/{index}.barcodeReadsCount.txt
barcodeStart=0
barcodeLen=24
umiStart=25
umiLen=10
mismatch=1
bcNum=38284877 ## Input the first line from output of CIDCount
polyAnum=15
mismatchInPolyA=2

```

### Run mapping pipeline

```

$ singularity exec SAW_v7.1.sif mapping \
    --outSAMattributes spatial \
    --outSAMtype BAM SortedByCoordinate \
    --genomeDir /path/to/genomeDir \
    --runThreadN 8 \
    --outFileNamePrefix /path/to/output/01.mapping/{index}. \ ## {in-
dex} or {idx} depending on the scenario
    --sysShell /bin/bash \
    --stParaFile /path/to/output/01.mapping/{index}.bcPara \ ## {in-
dex} or {idx} depending on the scenario
    --readNameSeparator \| \| \
    --limitBAMsortRAM 38582880124 \
    --limitOutSJcollapsed 10000000 \
    --limitIObufferSize=280000000 \
    --outBAMsortingBinsN 50 \
    > /path/to/output/01.mapping/{index}.run.log ## {index} or {idx}
depending on the scenario

```

## 2.3.3 Outputs

Q40 scenario 1 output files are organized as below:

```
$ tree /path/to/output/01.mapping/  
/path/to/output/01.mapping/  
├── lane.Aligned.sortedByCoord.out.bam  
├── lane.barcodeReadsCount.txt  
├── lane.bcPara  
├── lane.CIDMap.stat  
├── lane.Log.final.out  
├── lane.Log.out  
├── lane.Log.progress.out  
├── lane.run.log  
└── lane.SJ.out.tab
```

Q40 scenario 2 output files are organized as below (Here shows an example of 2 pairs of FASTQ):



**If one sample has multiple FASTQ files, you need to run `mapping` for each FASTQ pair.**

```
$ tree /path/to/multi_lane_output/01.mapping  
/path/to/multi_lane_output/01.mapping  
├── lane1.Aligned.sortedByCoord.out.bam  
├── lane1.barcodeReadsCount.txt  
├── lane1.bcPara  
├── lane1.CIDMap.stat  
├── lane1.Log.final.out  
├── lane1.Log.out  
├── lane1.Log.progress.out  
├── lane1.run.log  
├── lane1.SJ.out.tab  
├── lane2.Aligned.sortedByCoord.out.bam  
├── lane2.barcodeReadsCount.txt  
├── lane2.bcPara  
├── lane2.CIDMap.stat  
├── lane2.Log.final.out  
├── lane2.Log.out  
├── lane2.Log.progress.out  
├── lane2.run.log  
└── lane2.SJ.out.tab
```



Q4 scenario 1 (one barcode in library or did not split barcode when writing FASTQ) output files are organized as below:

```
$ tree /path/to/output/01.mapping
/path/to/output/01.mapping
├── 01.Aligned.sortedByCoord.out.bam
├── 01.barcodeReadsCount.txt
├── 01.bcPara
├── 01.CIDMap.stat
├── 01.Log.final.out
├── 01.Log.out
├── 01.Log.progress.out
├── 01.run.log
├── 01.SJ.out.tab
├── ...
├── 16.Aligned.sortedByCoord.out.bam
├── 16.barcodeReadsCount.txt
├── 16.bcPara
├── 16.CIDMap.stat
├── 16.Log.final.out
├── 16.Log.out
├── 16.Log.progress.out
├── 16.run.log
├── 16.SJ.out.tab
```

Q4 scenario 2 (multiple barcodes in the library and split when writing FASTQ) output files are organized as below (Here shows an example of 2 barcodes):

```
$ tree /path/to/output/01.mapping
/path/to/output/01.mapping
/path/to/output/01.mapping
├── 01.Aligned.sortedByCoord.out.bam
├── 01.barcodeReadsCount.txt
├── 01.bcPara
├── 01.CIDMap.stat
├── 01.Log.final.out
├── 01.Log.out
├── 01.Log.progress.out
├── 01.run.log
├── 01.SJ.out.tab
├── ...
├── 128.Aligned.sortedByCoord.out.bam
├── 128.barcodeReadsCount.txt
├── 128.bcPara
├── 128.CIDMap.stat
├── 128.Log.final.out
├── 128.Log.out
├── 128.Log.progress.out
├── 128.run.log
├── 128.SJ.out.tab
```

## 2.4 merge (optional)

SAW **merge** pipeline is used to combine the results of **mapping**.

Running **merge** requires the following file:

- **mapping** output mapped CID list files (**.txt**)
- 🕒 Expected running time for ~1G reads 2 lanes: ~5 min, Memory: ~5G

### 2.4.1 Arguments and Options

Table 2-5 merge Arguments and Options

| Parameter | Function  |
|-----------|---|
| [1]       | (Required) Path to the Stereo-seq Chip T mask file.             |
| [2]       | (Required) Mapped CID list files with read counts for each CID. |
| [3]       | (Required) Mapped CID list file which merges all input files.   |

### 2.4.2 Usage Example

```

$ mkdir /path/to/multi_lane_output/02.merge
$ singularity exec SAW_v7.1.sif merge \
  /path/to/data/{SN}.barcodeToPos.h5 \
  /path/to/multi_lane_output/01.mapping/{lane1}.barcodeReadsCount.txt, /path/to/
* multi_lane_output/01.mapping/{lane2}.barcodeReadsCount.txt \ ## change {lane} to
  {index} or {idx} for Q4 and change /path/to/multi_lane_output/ to /path/to/output/
  for SE single lane scenario
  /path/to/multi_lane_output/02.merge/{SN}.barcodeReadsCount.txt

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

### 2.4.3 Ouputs

The output file of **merge** has been organized as below:

```

$ tree /path/to/multi_lane_output/02.merge
/path/to/multi_lane_output/02.merge
├── {SN}.merge.barcodeReadsCount.txt

```

## 2.5 count

SAW **count** is an efficient general-purpose read annotation pipeline that label reads with their overlapped genomic features and outputs statistical information for the overall summarization result. Through quantification of annotated reads, **count** generates spatial gene expression data after de-duplicating reads according to CID, gene ID, and MID information. Usually, SAW **count** is run on the **Uniquely Mapped Reads** filtered from **mapping** output based on the reference genome annotation records. Starting from SAW v5.1.3, **count** allows the utilization of some Multi-Mapped Reads in quantification (`--multi_map`). The gene expression level in SAW pipeline is the summation of both intron and exon MID count. To support downstream analysis that might be required of different genomic features, **count** also outputs exon MID count separately.

Running **count** requires the following files:

- **mapping** output BAM file (**.bam**)
- Reference genome annotation GFF/GTF<sup>9,10</sup> file (**.gff / .gtf**)
- 🕒 Expected running time for ~1G reads: 0.5 h, Memory: ~45 G

### 2.5.1 Arguments and Options

Table 2-6 **count** Arguments and Options

| Parameter                | Function   |
|--------------------------|--|
| <code>-i</code>          | (Required) <b>mapping</b> output BAM file. Separate multiple files by comma.   |
| <code>-o</code>          | (Required) Set the <b>count</b> output BAM file name.  |
| <code>-a</code>          | (Required) Gene annotation GFF/GTF file.   |
| <code>-s</code>          | (Required) Set the <b>count</b> output statistical summary report file name.   |
| <code>-e</code>          | (Required) Set the <b>count</b> output gene expression file name.  |
| <code>--umi_len</code>   | (Required; defaults to 10) MID length.   |
| <code>--sn</code>        | (Required) Stereo-seq Chip T serial number (SN).   |
| <code>-c</code>          | (Optional; defaults to detected) CPU core number to use. Minimum value is 8, recommended value is 24.  |
| <code>--save_lq</code>   | (Optional; defaults to false) Save low-quality reads if set.   |
| <code>--save_dup</code>  | (Optional; defaults to false) Save duplicate reads if set.   |
| <code>--umi_on</code>    | (Optional; defaults to false) Correct MID if set.  |
| <code>--sat_file</code>  | (Optional; defaults to None) Set the saturation sampling file name which is prepared for sequencing saturation (requires <code>--umi_on</code> ).  |
| <code>--multi_map</code> | (Optional; defaults to disable) Set to enable multi-mapped reads correction. This correction consists of two logics. 1) The first logic is the correction of multi-gene reads which is a read mapped uniquely to a genomic region where multiple genes overlap. In this scenario, the read has to overlap with a genomic locus greater than 50% of its read length. If the genomic feature (exon, intron, or intergenic) of all the mapped genes includes exon and intron, then select the alignment record mapped to exon in preference to intron. If more than one gene locus belongs to the same genomic feature type, then pick the one that has the longest overlap. Otherwise, label the read to "intergenic." 2) The second logic is to select and correct one of the multi-mapped reads and add the count to gene expression matrix. The first step is to group reads by QNAME. Select reads in the group mapped to exon in preference to those mapped to intron. Then correct the longest overlapped reads (at least overlapped greater than 50% of its read length) in the group to unique read and correct its MAPQ to 255. Set the MAPQ of the remaining reads to 0. |

## 2.5.2 Usage Example

```

$ mkdir -p /path/to/output/03.count
$ geneExp=/path/to/output/03.count/{SN}.raw.gef
$ saturationSamplingFile=/path/to/output/03.count/{SN}_raw_barcode_gene_exp.txt
$ singularity exec SAW_v7.1.sif count \
    -i /path/to/output/01.mapping/{lane}.Aligned.sortedByCoord.out.bam \
    -o /path/to/output/03.count/{SN}.Aligned.sortedByCoord.out.merge.q10.dedup.
* target.bam \
    -a /path/to/reference/genes.gtf \
    -s /path/to/output/03.count/{SN}.Aligned.sortedByCoord.out.merge.q10.dedup.
* target.bam.summary.stat \
    -e ${geneExp} \
    --umi_len 10 \
    --sat_file ${saturationSamplingFile} \
    --sn {SN} \
    --umi_on \
    --save_lq \
    --save_dup \
    -c 24

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

For more than one pair of FASTQ files (Here shows an example of 2 pairs of FASTQ):

```

$ mkdir -p /path/to/multi_lane_output/03.count ## change /path/to/multi_lane_out-
put/ to /path/to/output/ for SE single lane scenario
$ geneExp=/path/to/multi_lane_output/03.count/{SN}.raw.gef
$ saturationSamplingFile=/path/to/multi_lane_output/03.count/{SN}_raw_barcode_gene_
exp.txt
$ singularity exec SAW_v7.1.sif count \
    -i /path/to/multi_lane_output/01.mapping/{lane1}.Aligned.sortedByCoord.out.
* bam,/path/to/ multi_lane_output/01.mapping/{lane2}.Aligned.sortedByCoord.out.
## change {lane} to {index} or {idx} for SE
    -o /path/to/multi_lane_output/03.count/{SN}.Aligned.sortedByCoord.out.
* merge.q10.dedup.target.bam \
    -a /path/to/reference/genes.gtf \
    -s /path/to/multi_lane_output/03.count/{SN}.Aligned.sortedByCoord.out.
* merge.q10.dedup.target.bam.summary.stat \
    -e ${geneExp} \
    --umi_len 10 \
    --sat_file ${saturationSamplingFile} \
    --sn {SN} \
    --umi_on \
    --save_lq \
    --save_dup \
    -c 24

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

For dealing with multi-mapped reads:

```
$ singularity exec SAW_v7.1.sif count \
  -i /path/to/output/01.mapping/{lane}.Aligned.sortedByCoord.out.bam \
  -o /path/to/output/03.count/{SN}.Aligned.sortedByCoord.out.merge.q10.dedup.
target.bam \
  -a /path/to/reference/genes.gtf \
  -s /path/to/output/03.count/{SN}.Aligned.sortedByCoord.out.merge.q10.dedup.
target.
bam.summary.stat \
  -e ${geneExp} \
  --umi_len 10 \
  --sat_file ${saturationSamplingFile} \
  --sn {SN} \
  --umi_on \
  --save_lq \
  --save_dup \
  -c 24 \
  --multi_map
```

### 2.5.3 Ouputs

The **count** output files are organized as below:

```
$ tree /path/to/output/03.count
/path/to/output/03.count
├── SN.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam
├── SN.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam.csi
├── SN.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam.summary.stat
├── SN_raw_barcode_gene_exp.txt
└── SN.raw.gef
```



When you run **mapping** using reference genome with rRNA information and the switch of **rRNAremove** is on, **PASS FILTER** item in **\*.bam.summary.stat** file will not contain the number of the reads mapped to rRNA reference.


## 2.6 register

SAW **register** pipeline aligns the microscopic tissue staining images with the plot of gene expression matrix generated by **count** based on the track lines on the chip while establishing the mapping relationship between images and spatial gene expression distribution. Staining images include:

- DAPI/ssDNA image for cell nucleus;
- IF (immunofluorescence) image for protein.
- H&E (hematoxylin and eosin) image for cell nuclei, extracellular matrix and cytoplasm.


SAW **register** includes four main modules, stitching, tissue segmentation, cell segmentation, and registration. Stitching combines microscopic images with overlapping sections to create a panoramic image (skip stitching if the input image is already a panoramic image). Tissue and cell segmentation modules detect and mask out tissue and cell coverage region, respectively. Registration module aligns the stitched image and the expression matrix, and at the same time registered masks from segmentation modules with the gene expression matrix using the same parameters. When it comes to the scenario of DAPI and mIF (multiple immunofluorescence images), this module outputs the registered DAPI and mIF image files. Tissue segmentation of IF image adopts the method of threshold segmentation, while cell segmentation of it is the intersection result of IF tissue segmentation, DAPI tissue segmentation and DAPI cell segmentation.

In addition, stitching and segmentation modules can be run separately with registration.

 **QC-failed data which is manually processed are supported currently. The tissue or cell segmentation image will adopt the manually processed results from ImageStudio (Both QC failure and success are accepted). More to the manual tutorial on GitHub.**

Running **register** requires the following files:

- **count** output gene expression matrix file (**.raw.gef**)
- The microscopic tissue staining image file (**.tar.gz**) processed by **ImageStudio**. **register** is compatible with QC outputs from **ImageStudio** v3.
- **ImageStudio** Image process record file (**.ipr**)
- 🕒 Expected running time for 1 cm x 1 cm Stereo-seq Chip T image: ~7.5 h, Memory: ~20 G

 **Cell segmentation is the most time-consuming part. You may use `-w False` to skip cell segmentation, but still perform stitching, tissue segmentation and registration.**

### 2.6.1 Arguments and Options

Table 2-7 **register** Arguments and Options

| Parameter | Function  |
|-----------|---|
| <b>-i</b> | (Required) ImageStudio processed staining image TAR.GZ file or image pre-processed output directory.  |
| <b>-c</b> | (Required) ImageStudio IPR (image process record) file. IPR is designed to efficiently hold images and process records generated from each image processing step along with metadata in various data types. Please check <a href="#">Stereo-seq File Format Manual</a> to get more information on the IPR file. |
| <b>-v</b> | (Optional. Depends on <b>-i</b> ) <b>count</b> output gene expression matrix GEF file.  |
| <b>-o</b> | (Required) Path to the directory where to store the <b>register</b> outputs.  |

| Parameter     | Function   |
|---------------|--|
| <b>-w</b>     | (Required, bool) Whether to do cell segmentation.  |
| <b>-P</b>     | (Optional, str=None) Customized IF thresholds and separated by comma and correspond to -g, e.g "-p 70,70". |
| <b>-g</b>     | (Optional, str=None) IF group names and separated by comma, e.g "-g 355_ID,648_IF".                        |
| <b>--gpu</b>  | (Optional, str=-1) Set GPU ID. eg: "--gpu 0". Default using CPU by "--gpu -1".                             |
| <b>--core</b> | (Optional, int=os.cpu_count()) Set CPU core number.  |



### Preparation of GPU Usage:

```
pip install onnxruntime-gpu==1.15.1
```

```
export LD_LIBRARY_PATH=/usr/local/cuda/lib64:$LD_LIBRARY_PATH
```

NVIDIA CUDA installation guide on Linux: <https://docs.nvidia.com/cuda/cuda-installation-guide-linux/>

## 2.6.2 Usage Example

Scenario 1: Process images from ImageStudio output data and gene expression matrix. Perform stitching, tissue segmentation, cell segmentation, and registration with gene expression matrix.

```
$ image=/path/to/data/image
$ image4register=$(find ${image} -maxdepth 1 -name {SN}*.tar.gz | head -1)
$ imageQC=$(find ${image} -maxdepth 1 -name {SN}*.ipr | head -1)
$ mkdir -p /path/to/output/04.register
$ singularity exec SAW_v7.1.sif register \
    -i ${image4register} \
    -c ${imageQC} \
    -v /path/to/output/03.count/{SN}.raw.gef \
    -o /path/to/output/04.register
    -w True ## do cell segmentation
```

Scenario 2: Process images from ImageStudio output data. Perform stitching, tissue segmentation, and cell segmentation without doing registration with the gene expression matrix.

```
$ image=/path/to/data/image
$ image4register=$(find ${image} -maxdepth 1 -name {SN}*.tar.gz | head -1)
$ imageQC=$(find ${image} -maxdepth 1 -name {SN}*.ipr | head -1)

$ mkdir -p /path/to/output/04.register
$ singularity exec SAW_v7.1.sif register \
    -i ${image4register} \
    -c ${imageQC} \
    -o /path/to/output/04.register
    -w True ## do cell segmentation
```

Scenario 3: Process images from scenario 2. register processed images with gene expression matrix.

```

$ imageQC=$(find /path/to/output/04.register -maxdepth 1 -name {SN}*.ipr | head -1)
$ mkdir -p /path/to/output/04.register
$ singularity exec SAW_v7.1.sif register \
  -i /path/to/output/04.register \ ## -i input scenario 2 output directory
  -c ${imageQC} \ ## scenario 2 output IPR
  -v /path/to/output/03.count/{SN}.raw.gef \
  -o /path/to/output/04.register
  -w False ## cell segmentation has been done in Scenario 2

```

## 2.6.3 Outputs

register output files of scenario 1 and scenario 3 (if `-i` and `-o` are identical) are organized as below:

```

$ tree /path/to/output/04.register
/path/to/output/04.register
... ## skip setting files, logs and image folder
├── <stainType>_fov_stitched.tif ## optional output when stainType=HE
├── <stainType>_fov_stitched_transformed.tif
└── SN_<chipType>_date_time_version.ipr

```

register output files of scenario 2:

```

$ tree /path/to/output/04.register
/path/to/output/04.register
... ## skip setting files, logs and image folder
├── <stainType>_fov_stitched.tif ## optional output when stainType=HE
├── <stainType>_fov_stitched_transformed.tif
└── SN_<chipType>_date_time_version.ipr

```

register output files of scenario 3 if `-i` and `-o` are two different paths:

```

$ tree /path/to/output/04.register
/path/to/output/04.register
... ## skip logs
└── SN_<chipType>_date_time_version.ipr

```

## 2.7 imageTools

SAW **imageTools** is a handy toolkit designed to play with image data in SAW. **imageTools ipr2img** (or **ipr2img**, available from SAW ST >= v5.0.0) is required in SAW core pipeline “decode” images from the processed IPR file. SAW **imageTools ipr2img** outputs TIFF images from IPR such as pre-registered ssDNA stitched image, tissue segmentation and cell segmentation mask TIFFs, as well as registered three types of images.



Running `imageTools ipr2img` requires the following files:

- ImageStudio processed microscopic tissue staining image file (**.tar.gz**)
- **register** processed Image process record file, supporting v0.2.1 (**.ipr**)
- 🕒 Expected running time for 1 cm x 1 cm Stereo-seq Chip T image: ~5 min, Memory: ~10 G

## 2.7.1 Arguments and Options

Table 2-8 `imageTools ipr2img` Arguments and Options

| Command              | Parameter       | Function   |
|----------------------|-----------------|--|
| <code>ipr2img</code> | <code>-i</code> | (Required) ImageStudio processed staining image TAR.GZ file.   |
|                      | <code>-c</code> | (Required) IPR (image process record) file. IPR is designed to efficiently hold image information generated from each processing step and datasets in various compound types along with metadata. Please check <a href="#">Stereo-seq File Format Manual</a> to get more information on IPR file.  |
|                      | <code>-d</code> | (Required) Segmentation module names. Convert segmentation mask TIFF from IPR for the selected modules. Valid options: <code>tissue</code> and <code>cell</code> . Separate modules by space.  |
|                      | <code>-r</code> | (Required; default to True) Whether output registered images or pre-registered images. “Pre-registered” state stands for the images that have been stitched to a single panoramic image and transformed to have the same scale with the gene expression matrix, but still need to be flipped, 90°rotated, or translated. The options are <code>True</code> for output registered images and <code>False</code> for output pre-registered images. |
|                      | <code>-o</code> | (Required) Path to the directory where to store the <code>ipr2img</code> outputs.  |



Please check [2.14.4 Other applications of imageTools](#) to learn more about `imageTools`.

## 2.7.2 Usage Example

```

$ image=/path/to/data/image
$ image4register=$(find ${image} -maxdepth 1 -name {SN}*.tar.gz | head -1)
$ imageIPR=$(find /path/to/output/04.register -maxdepth 1 -name {SN}*.ipr | head -1)
## has to be a processed IPR

$ singularity exec SAW_v7.1.sif imageTools ipr2img \
  -i ${image4register} \
  -c ${imageIPR} \
  -d tissue cell \ ## output both tissue and cell segmentation mask TIFF
  -r True \
  -o /path/to/output/04.register

```

## 2.7.3 Outputs

`imageTools ipr2img` output files (if the parent directory path of `-c` IPR and `-o` are identical) are organized as below:

```
$ tree /path/to/output/04.register
/path/to/output/04.register
... ## skip setting files, logs and image folder
├── <stainType>_fov_stitched.tif ## optional output when stainType=HE
├── <stainType>_fov_stitched_transformed.tif
├── <stainType>_matrix_template.txt
├── <stainType>_SN_mask.tif
├── <stainType>_SN_regist.tif
├── <stainType>_SN_tissue_cut.tif
├── <stainType>_transform_template.txt
├── fov_stitched_transformed.rpi
├── SN.rpi
└── SN_<chipType>_date_time_version.ipr
```

`imageTools ipr2img` output files (if the parent directory path of `-c` IPR and `-o` are two different paths) are organized as below:

```
$ tree /path/to/output/04.register_tmp
/path/to/output/04.register_tmp
... ## skip logs and image folder
├── <stainType>_fov_stitched.tif ## optional output when stainType=HE
├── <stainType>_fov_stitched_transformed.tif
├── <stainType>_matrix_template.txt
├── <stainType>_SN_mask.tif
├── <stainType>_SN_regist.tif
├── <stainType>_SN_tissue_cut.tif
├── <stainType>_transform_template.txt
└── SN.rpi
```

## 2.8 tissueCut

SAW `tissueCut` pipeline can delineate and extract the tissue coverage area based on the aligned image generated from `register` and `imageTools` or from the plot of gene expression matrix (if microscopic tissue staining images are not available). `tissueCut` outputs expression data in GEF format.



If the output of `tissueCut` doesn't match the morphology of the tissue, users could handle this issue with the help of `ImageStudio`, `StereoMap` or community developed tools, such as `Stereopy`<sup>11</sup>. If the `tissueCut` was run with an image, users could manually redo tissue segmentation for the image via `ImageStudio` and run `register`, `imageTools` and `tissueCut` again. Otherwise, manually delineate tissue coverage region from the gene expression distribution plot via `StereoMap` and then put the result into the SAW-lasso pipeline (2.15.6 lasso).

Running `tissueCut` requires the following files:

- Mapped CID list file (**.txt**)
- `count` output gene expression matrix file (**.raw.gef**)
- `imageTools ipr2img` output tissue segmentation mask TIFF file (**.tif, optional**)

⌚ Expected running time for ~1G reads: ~6 min, Memory: ~6 G



## 2.8.1 Arguments and Options

Table 2-9 `tissueCut` Arguments and Options

| Parameter               | Function   |
|-------------------------|--|
| <code>--dnbfile</code>  | (Optional) Mapped CID list file with reads counts for each CID. Input merged mapped CID list file if more than one list file was generated in <code>mapping</code> . |
| <code>-i</code>         | (Required) <code>count</code> output gene expression matrix file.  |
| <code>-o</code>         | (Required) Path to the directory where to store the <code>tissueCut</code> outputs.  |
| <code>-s</code>         | (Optional) Path to the <code>imageTools ipr2img</code> output tissue segmentation mask file. Only valid when <code>register</code> has been performed.               |
| <code>--sn</code>       | (Required) Stereo-seq Chip T serial number (SN).   |
| <code>--omics,-O</code> | (Required; default to Transcriptomics) String that specifies the omics.  |
| <code>-d</code>         | (Required) Set to generate required plots for <code>report</code> .  |
| <code>-t</code>         | (Optional) Number of threads used in numpy (recommended to fill 8 here).   |
| <code>-b</code>         | (Optional; default to 1,20,50,100,200) Set to specify bin sizes, separated by comma.   |
| <code>-l</code>         | (Optional) Set to specify the output label name.   |

## 2.8.2 Usage Example

Run `tissueCut` if `register` aligned microscopic staining image is provided:

- Generate tissue GEF based on the corresponding tissue mask image.

```
$ nucleusLayer=$(find /path/to/output/04.register -maxdepth 1 -name *fov_stitched_
transformed.tif -exec sh -c 'for f do basename -- "$f" _fov_stitched_transformed.
tif;done' sh {} + | grep -v IF)
$ tissueMaskFile=$(find /path/to/output/04.register -maxdepth 1 -name ${nucle-
usLayer}*_tissue_cut.tif)

$ mkdir -p /path/to/output/05.tissuecut
$ singularity exec SAW_v7.1.sif tissueCut \
  --dnbfile /path/to/output/02.merge/{SN}.merge.barcodeReadsCount.txt \
  -i /path/to/output/03.count/{SN}.raw.gef \
  -o /path/to/output/05.tissuecut \
  -s ${tissueMaskFile} \
  --sn {SN} -O Transcriptomics -d
```

- Output GEF with a customized label.

```
$ label=<labelName> ## customized label name
$ labelMaskFile=$(find /path/to/output/04.register -maxdepth 1 -name *${la-
bel}*_tissue_cut.tif)

$ mkdir -p /path/to/output/05.tissuecut/tissuecut_${label}
$ singularity exec SAW_v7.1.sif tissueCut \
  --dnbfile /path/to/output/02.merge/{SN}.merge.barcodeReadsCount.txt \
```

```

-i /path/to/output/03.count/{SN}.raw.gef \
-o /path/to/output/05.tissuecut/tissuecut_{label} \
-s ${labelMaskFile} \
-l ${label}
--sn {SN} -O Transcriptomics -d

```

Run `tissueCut` if image is not available:

```

$ mkdir -p /path/to/output/05.tissuecut
$ singularity exec SAW_v7.1.sif tissueCut \
  --dnbfile /path/to/output/02.merge/{SN}.merge.barcodeReadsCount.txt \
  -i /path/to/output/03.count/{SN}.raw.gef \
  -o /path/to/output/05.tissuecut \
  --sn {SN} -O Transcriptomics -d

```

## 2.8.3 Outputs

`tissueCut` output files:

Image is provided:

- Generate tissue GEF based on the corresponding tissue mask image.

```

$ tree /path/to/output/05.tissuecut
/path/to/output/05.tissuecut
├── SN.tissue.gef
├── tissuecut.stat
├── tissue_fig
│   ├── scatter_100x100_MID_gene_counts.png
│   ├── scatter_150x150_MID_gene_counts.png
│   ├── scatter_200x200_MID_gene_counts.png
│   ├── scatter_20X20_MID_gene_counts.png
│   ├── scatter_50x50_MID_gene_counts.png
│   ├── statistic_100x100_MID_gene_DNB.png
│   ├── statistic_150x150_MID_gene_DNB.png
│   ├── statistic_200x200_MID_gene_DNB.png
│   ├── statistic_20X20_MID_gene_DNB.png
│   ├── statistic_50x50_MID_gene_DNB.png
│   ├── violin_100x100_MID_gene.png
│   ├── violin_150x150_MID_gene.png
│   ├── violin_200x200_MID_gene.png
│   ├── violin_20X20_MID_gene.png
│   └── violin_50x50_MID_gene.png

```

- Output GEF with a customized label.

```

$ tree /path/to/output/05.tissuecut/tissuecut_<label>
/path/to/output/05.tissuecut/tissuecut_<label>
...
├── tissuecut_<label>
│   ├── SN.<label>.raw.label.gef
│   ├── <label>.tissuecut.stat
│   └── tissue_fig
│       ├── scatter_100x100_MID_gene_counts.png
│       ├── scatter_150x150_MID_gene_counts.png
│       ├── scatter_200x200_MID_gene_counts.png
│       ├── scatter_20x20_MID_gene_counts.png
│       ├── scatter_50x50_MID_gene_counts.png
│       ├── statistic_100x100_MID_gene_DNB.png
│       ├── statistic_150x150_MID_gene_DNB.png
│       ├── statistic_200x200_MID_gene_DNB.png
│       ├── statistic_20x20_MID_gene_DNB.png
│       ├── statistic_50x50_MID_gene_DNB.png
│       ├── violin_100x100_MID_gene.png
│       ├── violin_150x150_MID_gene.png
│       ├── violin_200x200_MID_gene.png
│       ├── violin_20x20_MID_gene.png
│       └── violin_50x50_MID_gene.png
...

```

Image is not provided:

```

$ tree /path/to/output/05.tissuecut
/path/to/output/05.tissuecut
├── 100X100_contour_image.png ## bin100 expression png
├── bin1_img.tif ## bin1 expresion distribution TIFF file
├── bin1_img_tissue_cut.tif ## tissue mask acquried from bin1 expression distribu-
tion plot
├── SN.tissue.gef
├── tissuecut.stat
└── tissue_fig
    ├── scatter_100x100_MID_gene_counts.png
    ├── scatter_150x150_MID_gene_counts.png
    ├── scatter_200x200_MID_gene_counts.png
    ├── scatter_20X20_MID_gene_counts.png
    ├── scatter_50x50_MID_gene_counts.png
    ├── statistic_100x100_MID_gene_DNB.png
    ├── statistic_150x150_MID_gene_DNB.png
    ├── statistic_200x200_MID_gene_DNB.png
    ├── statistic_20X20_MID_gene_DNB.png
    ├── statistic_50x50_MID_gene_DNB.png
    ├── violin_100x100_MID_gene.png
    ├── violin_150x150_MID_gene.png
    ├── violin_200x200_MID_gene.png
    ├── violin_20X20_MID_gene.png
    └── violin_50x50_MID_gene.png

```

## 2.9 spatialCluster

SAW **spatialCluster** pipeline performs clustering analysis for spots using community developed tools, such as stereopy. The clustering procedure includes 4 main steps: 1) preprocess gene expression data from the tissue-coverage region (normalize, logarithmize, identify highly-variable genes, and scale each gene), 2) reduce the dimensionality of the data by running PCA, 3) compute the neighborhood graph and embed neighborhood graph using UMAP, 4) clustering by Leiden algorithm.

Running **spatialCluster** requires the following files:

- **tissueCut** output GEF file for the tissue-covered region (**.tissue.gef**)
- 🕒 Expected running time for ~1G reads: ~1 min, Memory: ~5 G

### 2.9.1 Arguments and Options

Table 2-10 **spatialCluster** Arguments and Options

| Parameter | Function   |
|-----------|--|
| <b>-i</b> | (Required) <b>tissueCut</b> output GEF file for the tissue coverage area.  |
| <b>-o</b> | (Required) Output path for the clustering result in H5AD format.   |
| <b>-s</b> | (Required; default to 50) Bin size.  |
| <b>-r</b> | (Required; default to 1.0) Leiden resolution which controls the coarseness of the clustering. Higher values lead to more clusters. |

### 2.9.2 Usage Example

```

$ mkdir -p /path/to/output/06.spatialcluster
$ singularity exec SAW_v7.1.sif spatialCluster \
  -i /path/to/output/05.tissuecut/{SN}.tissue.gef \
  -r 1.0 \
  -s 200 \
  -o /path/to/output/06.spatialcluster/{SN}.spatial.cluster.h5ad
## SN is suffixed with bin_size and resolution information

```

### 2.9.3 Outputs

**spatialCluster** output files are:

```

$ tree /path/to/output/06.spatialcluster
/path/to/output/06.spatialcluster
├── SN.bin200_1.0.spatial.cluster.h5ad

```

## 2.10 cellCut

SAW **cellCut** pipeline runs to extract expression matrix of cell nucleus based on the aligned image generated from **register** and **imageTools**. **cellCut** outputs expression data in cell bin GEF format. Run **cellCut** if cell bin results are desired.

Running **cellCut** requires the following files:

- **count** output gene expression matrix file (**.raw.gef**)
- **register** and **imageTools** output cell segmentation mask TIFF file (**.tif**)

🕒 Expected running time for ~1G reads: ~2 min, Memory: ~10 G

### 2.10.1 Arguments and Options

Table 2-11 **cellCut** Arguments and Options

| Commands                    | Parameters   | Function  |
|-----------------------------|--|---|
| cgef                        | <b>-i, --input-file</b>                                      | (Required) Input GEF file.  |
|                             | <b>-m, --mask-file</b>                                       | (Required) Input cell segmentation mask file.                                 |
|                             | <b>-o, --output-file</b>                                     | (Required) Output cell bin GEF file.  |
|                             | <b>-b, --block</b>   | (Optional; default to 256,256) Block size.                                    |
|                             | <b>-r, --rand-celltype</b>                                   | (Optional; default to 0) Number of random cell type.                          |
|                             | <b>-t, --threads</b>   | (Optional; default to 1) Number of threads.                                   |
|                             | <b>-v, --verbose</b>   | (Optional) Verbose output.  |
|                             | <b>-n, --cnum</b>  | (Optional; default to 5000) Top level cell number.                            |
|                             | <b>-R, --ratio</b>   | (Optional; default to 20) Other level cell number ratio.                      |
|                             | <b>-a, --allocat</b>   | (Optional; default to 2) Allocation strategy.                                 |
|                             | <b>-g, --raw-gem</b>   | (Optional) Raw GEM file.  |
|                             | <b>-c, --canvas</b>  | (Optional; default to 0,0,90000,90000) Set canvas size, minX,minY,-maxX maxY. |
|                             | <b>-l, --limit</b>   | (Optional; default to 16,16) Set block limit.                                 |
|                             | <b>-S, --split</b>   | (Optional; default to 0) Split cellID to layers and blocks.                   |
| <b>-w, --errorCode-file</b> | (Optional; default to false) Set to turn on error code mode. |   |



Please check [2.14.1 Other applications of cellCut](#) to learn more about **cellCut**.

## 2.10.2 Usage Example

Run `cellCut` only if `register` aligned microscopic staining image is provided:

```
$ mkdir -p /path/to/output/051.cellcut
$ nucleusLayer=$(find /path/to/output/04.register -maxdepth 1 -name *fov_stitched_
transformed.tif -exec sh -c 'for f do basename -- "$f" _fov_stitched_transformed.
tif;done' sh {} + | grep -v IF)
$ nucleusMask=$(find /path/to/output/04.register -maxdepth 1 -name ${nucleusLayer}*
mask.tif)

$ singularity exec SAW_v7.1.sif cellCut cgef \
  -i /path/to/output/03.count/{SN}.raw.gef \
  -m ${nucleusMask} \
  -o /path/to/output/051.cellcut/{SN}.cellbin.gef
```

## 2.10.3 Outputs

`cellCut` output files:

```
$ tree /path/to/output/051.cellcut
/path/to/output/051.cellcut
├── SN.cellbin.gef
```

## 2.11 cellCorrect

SAW `cellCorrect` pipeline runs to make adjustments based on the aligned cell segmentation image generated from `register` and `imageTools` and extract expression matrix of the adjusted one. `cellCorrect` outputs expression data in cell bin GEF and GEM formats. Run `cellCorrect` if results of cell correction are desired.

Running `cellCorrect` requires the following files:

- `count` output gene expression matrix file (**.raw.gef**)
- `register` and `imageTools` output cell segmentation mask TIFF file (.tif)
- 🕒 Expected running time for ~1G reads: ~2 min, Memory: ~10 G

### 2.11.1 Arguments and Options

Table 2-12 `cellCluster` Arguments and Options

| Parameter       | Function  |
|-----------------|---|
| <code>-i</code> | (Required) Input GEF file.  |
| <code>-m</code> | (Required) Input cell segmentation mask file.   |
| <code>-d</code> | (Required; default to 10) Outspread distance based on the cellular contour of cell segmentation image, in pixels. |
| <code>-o</code> | (Required) Path to output cell bin GEF, GEM and adjusted mask files.  |



## 2.11.2 Usage Example

Run `cellCorrect` as below:

```
$ nucleusLayer=$(find /path/to/output/04.register -maxdepth 1 -name *fov_stitched_
transformed.tif -exec sh -c 'for f do basename -- "$f" _fov_stitched_
tif;done' sh {} + | grep -v IF)
$ nucleusMask=$(find /path/to/output/04.register -maxdepth 1 -name ${nucleusLayer}*
mask.tif)

$ singularity exec SAW_v7.1.sif cellCorrect \
  -i /path/to/output/03.count/{SN}.raw.gef \
  -m ${nucleusMask} \
  -d 10 \
  -o /path/to/output/051.cellcut
```

## 2.11.3 Outputs

`cellCorrect` output files:

```
$ tree /path/to/output/051.cellcut
/path/to/output/051.cellcut
├── SN.adjusted.cellbin.gef
├── <stainType>_SN_mask_edm_dis_10.tif
└── SN.adjusted.gem
```

## 2.12 cellCluster

SAW `cellCluster` pipeline runs by clustering cells using the Leiden algorithm similarly to the procedures of `spatialCluster`. Run `cellCluster` if cell bin results are desired.

Running `cellCluster` requires the following files:

- `cellCut` output cell bin gene expression matrix file (**.cellbin.gef**)
- 🕒 Expected running time for ~1G reads: ~5 min, Memory: ~5 G

### 2.12.1 Arguments and Options

Table 2-12 `cellCluster` Arguments and Options

| Parameter       | Function   |
|-----------------|--|
| <code>-i</code> | (Required) <code>cellCut</code> output cell bin gene expression matrix file. |
| <code>-o</code> | (Required) Output path to the clustering result in H5AD format.              |

## 2.12.2 Usage Example

```

$ mkdir -p /path/to/output/061.cellcluster
$ singularity exec SAW_v7.1.sif cellCluster \
  -i /path/to/output/051.cellcut/{SN}.adjusted.cellbin.gef \
  -o /path/to/output/061.cellcluster/{SN}.adjusted.cell.cluster.h5ad

$ singularity exec SAW_v7.1.sif cellCluster \
  -i /path/to/output/051.cellcut/{SN}.cellbin.gef \
  -o /path/to/output/061.cellcluster/{SN}.cell.cluster.h5ad

```

## 2.12.3 Outputs

cellCluster output files:

```

$ tree /path/to/output/061.cellcluster
/path/to/output/061.cellcluster
├── SN.adjusted.cell.cluster.h5ad
└── SN.cell.cluster.h5ad

```

## 2.13 saturation

SAW **saturation** pipeline is performed to compute the sequencing saturation for the tissue coverage area.

Running **saturation** requires the following files:

- **mapping** output statistical report of CID mapping (**.stat**)
- **count** output saturation sampling file (**.txt**)
- **count** output statistical report of annotation (**.stat**)
- **tissueCut** output GEF file for the tissue coverage area (**.tissue.gef**)

🕒 Expected running time for ~1G reads: ~5 min, Memory: ~5 G

### 2.13.1 Arguments and Options

Table 2-13 **saturation** Arguments and Options

| Parameter        | Function   |
|------------------|--|
| <b>-i</b>        | (Required) <b>count</b> output saturation sampling file.   |
| <b>--tissue</b>  | (Required) <b>tissueCut</b> output GEF file for the tissue coverage area.  |
| <b>-o</b>        | (Required) Path to the directory where to store the <b>saturation</b> outputs. There has to be an existing path. |
| <b>--bcstat</b>  | (Required) <b>mapping</b> output statistical report of CID mapping. Separate multiple files by comma.            |
| <b>--summary</b> | (Required) <b>count</b> output statistical report of annotation.   |

## 2.13.2 Usage Example

```

$ mkdir -p /path/to/output/07.saturation
$ singularity exec SAW_v7.1.sif saturation \
  -i /path/to/output/03.count/{SN}_raw_barcode_gene_exp.txt \
  --tissue /path/to/output/05.tissuecut/{SN}.tissue.gef \
  -o /path/to/output/07.saturation \
  --bcstat /path/to/output/01.mapping/{lane}.CIDMap.stat \
  --summary /path/to/output/03.count/{SN}.Aligned.sortedByCoord.out.merge.q10.
dedup.target.bam.summary.stat

```

\* Please be noted that these two lines belong to the same line of command.

For more than one pair of FASTQ files (Here showing an example of 2 pairs of FASTQ),

```

$ mkdir -p /path/to/multi_lane_output/07.saturation
$ singularity exec SAW_v7.1.sif saturation \
  -i /path/to/multi_lane_output/03.count/{SN}_raw_barcode_gene_exp.txt \
  --tissue /path/to/multi_lane_output/05.tissuecut/{SN}.tissue.gef \
  -o /path/to/multi_lane_output/07.saturation \
  --bcstat /path/to/multi_lane_output/01.mapping/{lane1}.CIDMap.stat,/path/to/
multi_lane_output/01.mapping/{lane2}.CIDMap.stat \
  --summary /path/to/multi_lane_output/03.count/{SN}.Aligned.sortedByCoord.out.
merge.q10.dedup.target.bam.summary.stat

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

## 2.13.3 Outputs

saturation output files:

```

$ tree /path/to/output/07.saturation
├── plot_1x1_saturation.png
├── plot_200x200_saturation.png
└── sequence_saturation.tsv

```

## 2.14 report

SAW **report** pipeline is performed to integrate analysis results from each step and generate the report in JSON format as well as a web report in HTML format. HTML analytical report integrate genes' spatial expression distribution, key statistical metrics, sequencing saturation plots, clustering analysis results, and image processing information. When it comes to the scenario of mIF, pseudo color (up to 7) is used on the bottom layer of clustering results.

Running **report** requires the following files and information:

- **mapping** output statistical report of CID mapping and **STAR** alignment (**.stat, .out**)
  - **count** output statistical report of annotation (**.stat**)
  - **register** processed image process record file and image pyramid RPI file (**.ipr, .rpi**)
  - **tissueCut** and **cellCut** (if available) output GEF file, statistical report of tissue-covered region, plots and image pyramid RPI file (**.gef, .stat, .png**)
  - **spatialCluster** and **cellCluster** (if available) output clustering H5AD file (**.h5ad**)
  - **saturation** output bin200 sequence saturation plot (**.png**)
  - species, tissue, and reference information
- 🕒 Expected running time for ~1G reads: ~1 min, Memory: 1G

### 2.14.1 Arguments and Options

Table 2-14 **report** Arguments and Options

| Parameter                 | Function   |
|---------------------------|--|
| <b>-m</b>                 | (Required) Statistical report of CID mapping. Separate multiple files by comma.  |
| <b>-a</b>                 | (Required) Statistical report of STAR alignment. Separate multiple files by comma.   |
| <b>-g</b>                 | (Required) Statistical report of annotation.   |
| <b>-l</b>                 | (Required) Statistical report of tissue-covered region.  |
| <b>-n</b>                 | (Required) GEF file that has wholeExp/bin200. Please check <a href="#">2.14.1 Other applications of cellCut</a> to get more information of GEF completion. |
| <b>-b</b>                 | (Required) <b>tissueCut</b> output bin 200 scatter plot.   |
| <b>-v</b>                 | (Required) <b>tissueCut</b> output bin 200 violin plot.  |
| <b>-c</b>                 | (Required) <b>tissueCut</b> output bin 200 statistics plot.  |
| <b>--bin20Saturation</b>  | (Required) <b>tissueCut</b> output bin 20 scatter plot.  |
| <b>--bin20violin</b>      | (Required) <b>tissueCut</b> output bin 20 violin plot.   |
| <b>--bin20MIDGeneDNB</b>  | (Required) <b>tissueCut</b> output bin 20 statistics plot.   |
| <b>--bin50Saturation</b>  | (Required) <b>tissueCut</b> output bin 50 scatter plot.  |
| <b>--bin50violin</b>      | (Required) <b>tissueCut</b> output bin 50 violin plot.   |
| <b>--bin50MIDGeneDNB</b>  | (Required) <b>tissueCut</b> output bin 50 statistics plot.   |
| <b>--bin100Saturation</b> | (Required) <b>tissueCut</b> output bin 100 scatter plot.   |
| <b>--bin100violin</b>     | (Required) <b>tissueCut</b> output bin 100 violin plot.  |
| <b>--bin100MIDGeneDNB</b> | (Required) <b>tissueCut</b> output bin 100 statistics plot.  |
| <b>--bin150Saturation</b> | (Required) <b>tissueCut</b> output bin 150 scatter plot.   |

| Parameter                  | Function  |
|----------------------------|---|
| <b>--bin150violin</b>      | (Required) tissueCut output bin 150 violin plot.  |
| <b>--bin150MIDGeneDNB</b>  | (Required) tissueCut output bin 150 statistics plot.  |
| <b>-d</b>                  | (Required) spatialCluster output H5AD file.   |
| <b>-t</b>                  | (Required) saturation output bin 200 sequence saturation plot.  |
| <b>-r standard_version</b> | (Required) Set to specifying report version.  |
| <b>-s</b>                  | (Required) The Stereo-seq Chip T serial number.   |
| <b>--pipelineVersion</b>   | (Required) Set to specifying analysis pipeline version.   |
| <b>-o</b>                  | (Required) The directory to store outputs.  |
| <b>--species</b>           | (Required) A string of species name.  |
| <b>--tissue</b>            | (Required) A string of tissue type.   |
| <b>-reference</b>          | (Required) A string of reference used for mapping   |
| <b>--rpi_resolution</b>    | (Optional; default to 100) The resolution of RPI. Valid options: <b>2, 10, 50, 100, 150.</b>  |
| <b>-i</b>                  | (Optional) The image pyramid RPI file.  |
| <b>--iprFile</b>           | (Optional) A processed IPR (image process record) file.   |
| <b>--cellBinGef</b>        | (Optional) The cell bin GEF file.   |
| <b>--cellCluster</b>       | (Optional) cellCluster output H5AD file.  |
| <b>--tissue_fig</b>        | (Optional) The directory of statistical images from tissueCut.  |
| <b>--qc_metrics</b>        | (Optional) The customized QC-metrics file (.xlsx) for SAW. QC-metrics focus on sequencing quality, and performance of in situ restoration of sequencing reads. For example, "Total Q30" and "Fraction MID in Spots Under Tissue". |

## 2.14.2 Usage Example

Run `report` if `register` aligned microscopic staining image is provided and has cell bin output:



**Please Note! Replace `{SN}`, `{lane}`, `{species_name}`, `{tissue_type}`, `{reference_index}` with the real information.**

```

...
$ imageIPR=$(find /path/to/output/04.register -maxdepth 1 -name {SN}*.ipr | head
-1) ## has to be a processed IPR
$ singularity exec SAW_v7.1.sif cellCut bgef \
  -i /path/to/output/03.count/{SN}.raw.gef \
  -o /path/to/output/05.tissuecut/{SN}.gef \
  -b 1,10,20,50,100,200,500 \
  -O Transcriptomics
* $ mkdir -p /path/to/output/08.report
$ singularity exec SAW_v7.1.sif report \
  -m /path/to/output/01.mapping/{lane}.CIDMap.stat \
  -a /path/to/output/01.mapping/{lane}.Log.final.out \
  -g /path/to/output/03.count/{SN}.Aligned.sortedByCoord.out.merge.q10.dedup.
target.bam.summary.stat \
  -l /path/to/output/05.tissuecut/tissuecut.stat \
  -n /path/to/output/05.tissuecut/{SN}.gef \

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

```

gene_counts.png \,--bin20violin,--bin20MIDGeneDNB
-d /path/to/output/06.spatialcluster/{SN}.bin200_1.0.spatial.cluster.h5ad \
-t /path/to/output/07.saturation/plot_200x200_saturation.png \
-b /path/to/output/05.tissuecut/tissue_fig/scatter_200x200_MID_gene_counts.
png \
-v /path/to/output/05.tissuecut/tissue_fig/violin_200x200_MID_gene.png \
-c /path/to/output/05.tissuecut/tissue_fig/statistic_200x200_MID_gene_DNB.
png \
--bin20Saturation /path/to/output/05.tissuecut/tissue_fig/scatter_20x20_MID_
gene_counts.png \
--bin20violin /path/to/output/05.tissuecut/tissue_fig/violin_20x20_MID_gene.
png \
--bin20MIDGeneDNB /path/to/output/05.tissuecut/tissue_fig/statistic_20x20_
MID_gene_DNB.png \
--bin50Saturation /path/to/output/05.tissuecut/tissue_fig/scatter_50x50_MID_
gene_counts.png \
--bin50violin /path/to/output/05.tissuecut/tissue_fig/violin_50x50_MID_gene.
png \
--bin50MIDGeneDNB /path/to/output/05.tissuecut/tissue_fig/statistic_50x50_
MID_gene_DNB.png \
--bin100Saturation /path/to/output/05.tissuecut/tissue_fig/scatter_100x100_
MID_gene_counts.png \
--bin100violin /path/to/output/05.tissuecut/tissue_fig/violin_100x100_MID_
gene.png \
--bin100MIDGeneDNB /path/to/output/05.tissuecut/tissue_fig/statis-
tic_100x100_MID_gene_DNB.png \
--bin150Saturation /path/to/output/05.tissuecut/tissue_fig/scatter_150x150_
MID_gene_counts.png \
--bin150violin /path/to/output/05.tissuecut/tissue_fig/violin_150x150_MID_
gene.png \
--bin150MIDGeneDNB /path/to/output/05.tissuecut/tissue_fig/statis-
tic_150x150_MID_gene_DNB.png \
--cellBinGef /path/to/output/051.cellcut/{SN}.adjusted.cellbin.gef \
--cellCluster /path/to/output/061.cellcluster/{SN}.adjusted.cell.cluster.h5ad \
-i /path/to/output/04.register/{SN}.rpi \
-r standard_version \
-s {SN} \
--pipelineVersion SAW_v7.0.0 \
--iprFile ${imageIPR} \
--species {species_name} \
--tissue {tissue_type} \
--reference {reference_index} \
-o /path/to/output/08.report

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

For more than one pair of FASTQ files (Here showing an example of 2 pairs of FASTQ),

```

...
$ mkdir -p /path/to/multi_lane_output/08.report
$ singularity exec SAW_v7.1.sif cellCut bgef \
  -i /path/to/output/03.count/{SN}.raw.gef \
  -o /path/to/output/05.tissuecut/{SN}.gef \
  -b 1,10,20,50,100,200,500 \
  -O Transcriptomics
$ singularity exec SAW_v7.1.sif report \
  -m /path/to/multi_lane_output/01.mapping/{lane1}.CIDMap.stat,/path/to/multi_
  lane_output/01.mapping/{lane2}.CIDMap.stat \
  -a /path/to/multi_lane_output/01.mapping/{lane1}.Log.final.out,/path/to/
  multi_lane_output/01.mapping/{lane2}.Log.final.out \
  -g /path/to/multi_lane_output/03.count/{SN}.Aligned.sortedByCoord.out.merge.
  q10.dedup.target.bam.summary.stat \
  -l /path/to/multi_lane_output/05.tissuecut/tissuecut.stat \
  -n /path/to/multi_lane_output/05.tissuecut/{SN}.gef \
  -d /path/to/multi_lane_output/06.spatialcluster/{SN}.bin200_1.0.spatial.
  cluster.h5ad \
  -t /path/to/multi_lane_output/07.saturation/plot_200x200_saturation.png \
  -b /path/to/multi_lane_output/05.tissuecut/tissue_fig/scatter_200x200_MID_
  gene_counts.png \
  -v /path/to/multi_lane_output/05.tissuecut/tissue_fig/violin_200x200_MID_
  gene.png \
  -c /path/to/multi_lane_output/05.tissuecut/tissue_fig/statistic_200x200_MID_
  gene_DNB.png \
  --bin20Saturation /path/to/multi_lane_output/05.tissuecut/tissue_fig/scat-
  ter_20x20_MID_gene_counts.png \
  --bin20violin /path/to/multi_lane_output/05.tissuecut/tissue_fig/vio-
  lin_20x20_MID_gene.png \
  --bin20MIDGeneDNB /path/to/multi_lane_output/05.tissuecut/tissue_
  fig/statistic_20x20_MID_gene_DNB.png \
  --bin50Saturation /path/to/multi_lane_output/05.tissuecut/tissue_fig/scat-
  ter_50x50_MID_gene_counts.png \
  --bin50violin /path/to/multi_lane_output/05.tissuecut/tissue_fig/vio-
  lin_50x50_MID_gene.png \
  --bin50MIDGeneDNB /path/to/multi_lane_output/05.tissuecut/tissue_
  fig/statistic_50x50_MID_gene_DNB.png \
  --bin100Saturation /path/to/multi_lane_output/05.tissuecut/tissue_
  fig/scatter_100x100_MID_gene_counts.png \
  --bin100violin /path/to/multi_lane_output/05.tissuecut/tissue_
  fig/violin_100x100_MID_gene.png \
  --bin100MIDGeneDNB /path/to/multi_lane_output/05.tissuecut/tissue_
  fig/statistic_100x100_MID_gene_DNB.png \
  --bin150Saturation /path/to/multi_lane_output/05.tissuecut/tissue_
  fig/scatter_150x150_MID_gene_counts.png \

```

```

--bin150violin /path/to/multi_lane_output/05.tissuecut/tissue_
fig/violin_150x150_MID_gene.png \
--bin150MIDGeneDNB /path/to/multi_lane_output/05.tissuecut/tissue_
fig/statistic_150x150_MID_gene_DNB.png \
--cellBinGef /path/to/output/051.cellcut/{SN}.adjusted.cellbin.gef \
--cellCluster /path/to/output/061.cellcluster/{SN}.adjusted.cell.cluster.h5ad
-i /path/to/multi_lane_output/04.register/{SN}.rpi \
-r standard_version \
-s {SN} \
--pipelineVersion SAW_v7.0.0 \
--iprFile ${imageIPR} \
--species {species_name} \
--tissue {tissue_type} \
--reference {reference_index} \
-o /path/to/multi_lane_output/08.report

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

Run **report** of outputs in which the microscopic image has been registered with gene expression matrix but did not perform cell segmentation. Here is an example of just one pair of FASTQ,

```

$ mkdir -p /path/to/output/08.report
$ singularity exec SAW_v7.0.0.sif cellCut bgef \
-i /path/to/output/03.count/{SN}.raw.gef \
-o /path/to/output/05.tissuecut/{SN}.gef \
-b 1,10,20,50,100,200,500 \
-O Transcriptomics

$ singularity exec SAW_v7.0.0.sif report \
-m /path/to/output/01.mapping/{lane}.CIDMap.stat \
-a /path/to/output/01.mapping/{lane}.Log.final.out \
-g /path/to/output/03.count/{SN}.Aligned.sortedByCoord.out.merge.q10.dedup.
target.bam.summary.stat \
-l /path/to/output/05.tissuecut/tissuecut.stat \
-n /path/to/output/05.tissuecut/{SN}.gef \
-d /path/to/output/06.spatialcluster/{SN}.bin200_1.0.spatial.cluster.h5ad \
-t /path/to/output/07.saturation/plot_200x200_saturation.png \
-b /path/to/output/05.tissuecut/tissue_fig/scatter_200x200_MID_gene_counts.png
\
-v /path/to/output/05.tissuecut/tissue_fig/violin_200x200_MID_gene.png \
-c /path/to/output/05.tissuecut/tissue_fig/statistic_200x200_MID_gene_DNB.png
\
--bin20Saturation /path/to/output/05.tissuecut/tissue_fig/scatter_20x20_MID_
gene_counts.png \
--bin20violin /path/to/output/05.tissuecut/tissue_fig/violin_20x20_MID_gene.
png \
--bin20MIDGeneDNB /path/to/output/05.tissuecut/tissue_fig/statistic_20x20_MID_
gene_DNB.png \

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.



```

--bin50Saturation /path/to/output/05.tissuecut/tissue_fig/scatter_50x50_MID_
gene_counts.png \
--bin50violin /path/to/output/05.tissuecut/tissue_fig/violin_50x50_MID_gene.
png \
--bin50MIDGeneDNB /path/to/output/05.tissuecut/tissue_fig/statistic_50x50_MID_
gene_DNB.png \
--bin100Saturation /path/to/output/05.tissuecut/tissue_fig/scatter_100x100_
MID_gene_counts.png \
--bin100violin /path/to/output/05.tissuecut/tissue_fig/violin_100x100_MID_
gene.png \
--bin100MIDGeneDNB /path/to/output/05.tissuecut/tissue_fig/statistic_100x100_
MID_gene_DNB.png \
--bin150Saturation /path/to/output/05.tissuecut/tissue_fig/scatter_150x150_
MID_gene_counts.png \
--bin150violin /path/to/output/05.tissuecut/tissue_fig/violin_150x150_MID_gene.
png \
--bin150MIDGeneDNB /path/to/output/05.tissuecut/tissue_fig/statistic_150x150_
MID_gene_DNB.png \
-i /path/to/output/04.register/{SN}.rpi \
-r standard_version \
-s {SN} \
--pipelineVersion SAW_v7.0.0 \
--iprFile ${imageIPR} \
--species {species_name} \
--tissue {tissue_type} \
--reference {reference_index} \
-o /path/to/output/08.report

```

Run **report** for **register** aligned microscopic staining image is absent (Here shows an example of just one pair of FASTQ, similar to multiple pairs),

```

$ mkdir -p /path/to/output/08.report
$ singularity exec SAW_v7.1.sif cellCut bgef \
-i /path/to/output/03.count/{SN}.raw.gef \
-o /path/to/output/05.tissuecut/{SN}.gef \
-b 1,10,20,50,100,200,500 \
-O Transcriptomics
$ singularity exec SAW_v7.1.sif report \
-m /path/to/output/01.mapping/{lane}.CIDMap.stat \
-a /path/to/output/01.mapping/{lane}.Log.final.out \
-g /path/to/output/03.count/{SN}.Aligned.sortedByCoord.out.merge.q10.dedup.
target.bam.summary.stat \

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

```

-l /path/to/output/05.tissuecut/tissuecut.stat \
-n /path/to/output/05.tissuecut/{SN}.gef \
-d /path/to/output/06.spatialcluster/{SN}.bin200_1.0.spatial.cluster.h5ad \
-t /path/to/output/07.saturation/plot_200x200_saturation.png \
-b /path/to/output/05.tissuecut/tissue_fig/scatter_200x200_MID_gene_counts.
png \
-v /path/to/output/05.tissuecut/tissue_fig/violin_200x200_MID_gene.png \
-c /path/to/output/05.tissuecut/tissue_fig/statistic_200x200_MID_gene_DNB.
png \
--bin20Saturation /path/to/output/05.tissuecut/tissue_fig/scatter_20x20_MID_
gene_counts.png \
--bin20violin /path/to/output/05.tissuecut/tissue_fig/violin_20x20_MID_gene.
png \
--bin20MIDGeneDNB /path/to/output/05.tissuecut/tissue_fig/statistic_20x20_
MID_gene_DNB.png \
--bin50Saturation /path/to/output/05.tissuecut/tissue_fig/scatter_50x50_MID_
gene_counts.png \
--bin50violin /path/to/output/05.tissuecut/tissue_fig/violin_50x50_MID_gene.
png \
--bin50MIDGeneDNB /path/to/output/05.tissuecut/tissue_fig/statistic_50x50_
MID_gene_DNB.png \
--bin100Saturation /path/to/output/05.tissuecut/tissue_fig/scatter_100x100_
MID_gene_counts.png \
--bin100violin /path/to/output/05.tissuecut/tissue_fig/violin_100x100_MID_
gene.png \
--bin100MIDGeneDNB /path/to/output/05.tissuecut/tissue_fig/statis-
tic_100x100_MID_gene_DNB.png \
--bin150Saturation /path/to/output/05.tissuecut/tissue_fig/scatter_150x150_
MID_gene_counts.png \
--bin150violin /path/to/output/05.tissuecut/tissue_fig/violin_150x150_MID_
gene.png \
--bin150MIDGeneDNB /path/to/output/05.tissuecut/tissue_fig/statis-
tic_150x150_MID_gene_DNB.png \
-r standard_version \
-s {SN} \
--pipelineVersion SAW_v7.0.0 \
--species {species_name} \
--tissue {tissue_type} \
--reference {reference_index} \
-o /path/to/output/08.report

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

### 2.14.3 Outputs

**report** output files that have cell bin data input are organized as below:

```
$ tree /path/to/output/08.report
/path/to/output/08.report
├── scatter_1x1_MID_gene_counts.png
├── SN.report.html
├── SN.statistics.json
├── statistic_1x1_cell_area.png
├── statistic_1x1_DNB.png
├── statistic_1x1_gene.png
├── statistic_1x1_MID.png
├── violin_1x1_gene.png
└── violin_1x1_MID.png
```

**report** output files that the cell bin data inputs are absent:

```
$ tree /path/to/output/08.report
/path/to/output/08.report
├── SN.report.html
└── SN.statistics.json
```

## 2.15 Other Applications

### 2.15.1 Other applications of cellCut

SAW **cellCut** is also an application for manipulating GEF file. SAW contains this tool to convert GEF format gene expression matrix to plain table or complete a GEF. Users may also manipulate the GEF files using the separate individual C++ compiled program `gefTools`<sup>12</sup> or its python encapsulated package `gefpy`<sup>13</sup>.

## 2.15.1.1 Arguments and Options

Table 2-15 Other applications of cellCut Arguments and Options

| Commands                    | Parameters   | Function   |
|-----------------------------|--|--|
| view                        | <b>-i, --input-file</b>                                      | (Required) Input bin GEF file or cell bin GEF file.  |
|                             | <b>-o, --output-file</b>                                     | (Optional; default to stdout) Output GEM file.   |
|                             | <b>-d, --exp_data</b>  | (Optional; default to "") Input bin1 GEF to get cell bin GEM.  |
|                             | <b>-m, --mask-file</b>                                       | (Optional; default to "") Input cell segmentation mask file, when cell bin GEM is expected output.   |
|                             | <b>-b, --bin-size</b>  | (Optional; default to 1) Set bin size for bin GEF file. Only valid for bin GEF.  |
|                             | <b>-s, --serial-number</b>                                   | (Required) Stereo-seq Chip T serial number.  |
|                             | <b>-e, --exon</b>  | (Optional; default to 1) Whether or not output exon group.   |
| bgef                        | <b>-w, --errorCode-file</b>                                  | (Optional; default to false) Determine output error code to file.  |
|                             | <b>-i, --input-file</b>                                      | (Required) Input gene expression matrix file (.gem/.gem.gz) or bin1 GEF file.  |
|                             | <b>-o, --output-file</b>                                     | (Required) Output bin GEF file.  |
|                             | <b>-b, --bin-size</b>  | (Required; default to 1,10,20,50,100,200,500) Comma-separate bin size list.  |
|                             | <b>-r, --region</b>  | (Optional; default to "") A rectangular region represented by the comma-separated list of vertex coordinates. For example, minX,maxX-,minY,maxY.     |
|                             | <b>-t, --threads</b>   | (Optional; default to 8) Number of threads.  |
|                             | <b>-s, --stat</b>  | (Optional; default to true) Whether create stat group. Stat group includes a gene dataset which contains total MIDcount and E10 score for each gene. |
| cgef                        | <b>-O, --omics</b>   | (Required; default to Transcriptomics) Specify the omics.  |
|                             | <b>-v, --verbose</b>   | (Optional) Verbose output.   |
|                             | <b>-w, --errorCode-file</b>                                  | (Optional; default to false) Determine output error code to file.  |
|                             | <b>-i, --input-file</b>                                      | (Required) Input GEF file.   |
|                             | <b>-m, --mask-file</b>                                       | (Required) Input cell segmentation mask file.  |
|                             | <b>-o, --output-file</b>                                     | (Required) Output cell bin GEF file.   |
|                             | <b>-b, --block</b>   | (Optional; default to 256,256) Block size.   |
|                             | <b>-r, --rand-celltype</b>                                   | (Optional; default to 0) Number of random cell type.   |
|                             | <b>-t, --threads</b>   | (Optional; default to 1) Number of threads.  |
|                             | <b>-v, --verbose</b>   | (Optional) Verbose output.   |
|                             | <b>-n, --cnum</b>  | (Optional; default to 5000) Top level cell number.   |
|                             | <b>-R, --ratio</b>   | (Optional; default to 20) Other level cell number ratio.   |
|                             | <b>-a, --allocat</b>   | (Optional; default to 2) Allocation strategy.  |
|                             | <b>-g, --raw-gem</b>   | (Optional) Raw GEM file.   |
|                             | <b>-c, --canvas</b>  | (Optional; default to 0,0,90000,90000) Set canvas size, minX, minY, maxX, maxY.  |
| <b>-l, --limit</b>          | (Optional; default to 16,16) Set block limit.                |  |
| <b>-S, --split</b>          | (Optional; default to 0) Split cellID to layers and blocks.  |  |
| <b>-w, --errorCode-file</b> | (Optional; default to false) Set to turn on error code mode. |  |



## 2.15.1.2 Usage Examples

Function 1: GEF to plain table GEM format

```

...
$ singularity exec SAW_v7.1.sif cellCut view \  ## convert GEF that only contains
bin1 geneExp
  -s {SN} \
  -i /path/to/output/03.count/{SN}.raw.gef \
  -o {SN}.raw.gem
$ singularity exec SAW_v7.1.sif cellCut view \  ## convert a whole GEF
  -s {SN} \
  -i /path/to/output/05.tissuecut/{SN}.gef \
  -o {SN}.gem
$ singularity exec SAW_v7.1.sif cellCut view \  ## convert tissue GEF that only
contains bin1 geneExp
  -s {SN} \
  -i /path/to/output/05.tissuecut/{SN}.tissue.gef \
  -o {SN}.tissue.gem
$ singularity exec SAW_v7.1.sif cellCut view \  ## convert cellbin GEF to cellbin
GEM
  -s {SN} \
  -i /path/to/output/051.cellcut/{SN}.cellbin.gef \
  -o {SN}.cellbin.gem \
  -d /path/to/output/03.count/{SN}.raw.gef

```

Function 2: completion of a GEF

```

...
$ singularity exec SAW_v7.1.sif cellCut bgef \  ## complete GEF that only contains
bin1 geneExp group to a whole GEF, you may specify the bin size using "-b". Sepa-
rate multiple bin size with comma
  -i /path/to/output/03.count/{SN}.tissue.gef \
  -o {SN}.tissue.complete.gef \
  -b 1,20,50,100 \
  -O Transcriptomics

```

Function 3: converting GEM to GEF

```

...
$ singularity exec SAW_v7.1.sif cellCut bgef \  ## convert GEM to GEF in specific
bin size. Separate multiple bin sizes with comma
  -i {SN}.gem \
  -o {SN}.gef \
  -b 1,20,50 \
  -O Transcriptomics

```

Example of GEF to GEM conversion using `gefpy`, users may specify the bin size.

```

...

```

## 2.15.2 checkGTF

SAW **checkGTF** is an application for checking whether the GTF/GFF file is in the correct format as the input for **count**, especially the limitation to the length of gene names.

Running **checkGTF** requires the following files:

- Reference genome annotation GFF/GTF<sup>9,10</sup> file (**.gff / .gtf**)

### 2.15.2.1 Arguments and Options

Table 2-17 **checkGTF** Arguments and Options

| Parameter | Function                                    |
|-----------|---|
| <b>-i</b> | (Required) Gene annotation GFF/GTF file.    |
| <b>-o</b> | (Required) Output a re-format GFF/GTF file. |

### 2.15.2.2 Usage Examples

```
$ singularity exec SAW_v7.1.sif checkGTF \  
-i /path/to/reference/genes.gtf \  
-o /path/to/reference/genes_new.gtf \  

```

## 2.15.3 Other applications of imageTools

Besides **ipr2img**, **imageTools** has three more functions: **img2rpi** (writes images in RPI format), **merge** (merges stain image with tissue segmentation and cell segmentation images to check the segmentation result), and **overlay** (stacks inferred track line template with stitched panoramic image to check stitching result or stacks track line template from matrix with registered panoramic image to check registration result).

### 2.15.3.1 Arguments and Options

Table 2-18 Other applications of `imageTools` Arguments and Options

| Commands       | Parameters | Function  |
|----------------|------------|---|
| <b>img2rpi</b> | <b>-i</b>  | (Required) Input TIFF file. Separate multiple files by comma.   |
|                | <b>-g</b>  | (Required) Set the '<stainType>/<imageType>' of the input TIFF stores in the RPI. Separate multiple files by comma, the order of groups needs to be exactly same with the input TIFF files.   |
|                | <b>-b</b>  | (Required) Bin sizes. Separate multiple bin sizes with space, for example 1 10 50 100   |
|                | <b>-o</b>  | (Required) Output path for the RPI file. Has to use absolute path or relative path.   |
|                | <b>-c</b>  | (Optional) Customized mask colors in RGB format only valid for (<stainType>/TissueMask_<label>/Color. The default color is set to be (255,255,255) for one mask. Separate multiple mask colors by comma and add escape characters before brackets.<br>For example: -c \ (10,20,30\),\ (30,40,50\).  |
| <b>merge</b>   | <b>-i</b>  | (Required) Input TIFF files. Separate multiple files by comma. The maximum number of TIFF files to be composited is three, and the channel order is R-G-B.  |
|                | <b>-o</b>  | (Required) Output path for the multichannel image.  |
| <b>overlay</b> | <b>-i</b>  | (Required) Input TIFF file. Usually input a pre-registered panoramic image if overlaid with the stitching template derived from track cross, and input a registered microscopic image if overlaid with the track line template acquired from gene expression matrix.  |
|                | <b>-c</b>  | (Required) Image configuration file (.ipr) or template points file (.txt).  |
|                | <b>-o</b>  | (Required) Output path of the TIFF file which has the template overlaid on the selected image.  |
|                | <b>-d</b>  | (Required) Module name. Convert template from IPR for the selected modules. Valid options: <b>stitch</b> or <b>register</b> .   |
|                | <b>-l</b>  | (Optional, default to 30) Cross limbs length in pixel.  |
|                | <b>-w</b>  | (Optional, default to 1) Cross line thickness in pixel.   |
| <b>ipr2img</b> | <b>-i</b>  | (Required) ImageStudio processed staining image TAR.GZ file.  |
|                | <b>-c</b>  | (Required) IPR (image process record) file. IPR is designed to efficiently hold image information generated from each processing step and datasets in various compound types along with metadata. Please check <a href="#">Stereo-seq File Format Manual</a> to get more information on IPR file.   |
|                | <b>-d</b>  | (Required) Segmentation module names. Convert segmentation mask TIFF from IPR for the selected modules. Valid options: <b>tissue</b> and <b>cell</b> . Separate modules by space.   |
|                | <b>-r</b>  | (Required; default to True) Whether output registered images or pre-registered images. "Pre-registered" state stands for the images that have been stitched to a single panoramic image and transformed to have the same scale with the gene expression matrix, but still need to be flipped, rotated, or translated. The options are <b>True</b> for output registered images and <b>False</b> for output pre-registered images. |
|                | <b>-o</b>  | (Required) Path to the directory where to store the <code>imageTools ipr2img</code> outputs.  |



### 2.15.3.2 Usage Examples

#### Function 1: `img2rpi`

```

$ singularity exec SAW_v7.1.sif imageTools img2rpi \
  -i /path/to/output/04.register/<stainType1>_fov_stitched_transformed.tif,
/path/to/output/04.register/<stainType2>_fov_stitched_transformed.tif \
  -g <stainType1>/<ImageType>,<stainType2>/<ImageType>\
  -b 1 10 50 100 \
  -o /path/to/output/04.register/fov_stitched_transformed.rpi  ## this RPI is
used for manual registration in StereoMap

```

```

# An easy example of transforming multiple <stainType>_fov_stitched_transformed.tif
# and storing as '<stainType>/Image' into fov_stitched_transformed.rpi

$ registerTif=$(find /path/to/output/04.register -maxdepth 1 -name \
*fov_stitched_transformed.tif)
$ regTifStr=$(echo $registerTif | tr ' ' ',')
$ regGroup=$(find /path/to/output/04.register -maxdepth 1 -name \
'*fov_stitched_transformed.tif' -exec sh -c 'for f do basename -- "$f" \
_fov_stitched_transformed.tif;done' sh {} +)
$ regGroupStr=$(echo $regGroup | sed 's/ \|$\/Image,/g' | sed 's/.$//') \

$ singularity exec SAW_v7.1.sif imageTools img2rpi \
  -i ${regTifStr} \
  -g ${regGroupStr} \ ## <stainType>/Image ,<stainType>/Image
  -b 1 10 50 100 \
  -o /path/to/output/04.register/fov_stitched_transformed.rpi

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

```

## Cutomize labeled-tissue-mask color

$ singularity exec SAW_v7.1.sif imageTools img2rpi \
  -i /path/to/<stainType>_{SN}_<label01>_tissue_cut.tif,/path/to/<stainType>_{SN}
_<label02>_tissue_cut.tif\
  -g <stainType>/TissueMask_<label01>,<stainType>/TissueMask_<label02> \
  -b 1 10 50 100 \
  -c \ (100,200,255\),\ (200,25,255\ ) \ ## escape character "\" before brackets
  -o /path/to/fov_stitched.rpi  ## this RPI could be used for manual registration
in StereoMap

```



Function 2: `merge` (`-i` Arbitrary up to three grayscale images (`.tif`))

```

$ singularity exec SAW_v7.1.sif imageTools merge \
  -i /path/to/output/04.register/<stainType>_{SN}_regist.tif,/path/to/output/04.
* register/<stainType>_{SN}_tissue_cut.tif,/path/to/output/04.register/<stainType>_
  {SN}_mask.tif \
  -o /path/to/output/04.register/merge.tif

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

Function 3: `overlay`

Overlay stitching template in IPR or in a TXT format with the pre-registered panoramic image to check the stitching result.

```

## stitch
preRegImage=/path/to/output/04.register/<stainType>_fov_stitched_transformed.tif
imageIPR=$(find /path/to/output/04.register -maxdepth 1 -name {SN}*.ipr | head -1)
stitchTplt=/path/to/output/04.register/<stainType>_transform_template.txt

singularity exec SAW_v7.1.sif imageTools overlay \
  -i ${preRegImage} \
  -c ${imageIPR} \
  -o /path/to/output/04.register/<stainType>_stitch_check.tif \
  -d stitch \
  -l 60 \
  -w 3

singularity exec SAW_v7.1.sif imageTools overlay \
  -i ${preRegImage} \
  -c ${stitchTplt} \
  -o /path/to/output/04.register/<stainType>_stitch_check_tmplt.tif \
  -d stitch \
  -l 60 \
  -w 3

```

Overlay registration template in IPR or in a TXT format with the registered panoramic image.

```

### register
$ regImage=/path/to/output/04.register/<stainType>_{SN}_regist.tif
imageIPR=$(find /path/to/output/04.register -maxdepth 1 -name {SN}*.ipr | head -1)
regTplt=/path/to/output/04.register/<stainType>_matrix_template.txt

$ singularity exec SAW_v7.1.sif imageTools overlay \
  -i ${regImage} \
  -c ${imageIPR} \
  -o /path/to/output/04.register/register_check.tif \
  -d register \
  -l 60 \
  -w 3

$ singularity exec SAW_v7.1.sif imageTools overlay \
  -i ${regImage} \
  -c ${regTplt} \
  -o /path/to/output/04.register/register_check_tplt.tif \
  -d register \
  -l 60 \
  -w 3

```

#### Function 4: `ipr2img`

Output images (.tif) from IPR file. Other usages of this function can be found in the tutorial for manual processing workflow on GitHub (<https://github.com/STOmics/SAW/blob/main/Documents/UserManual/>).

### 2.15.4 manualRegister

SAW manualRegister pipeline is performed when automatic registration result does not meet the requirement. Users can use **StereoMap**, an offline visualization software, to manually register images with the gene expression matrix. The operation parameters are recorded in the JSON file and can be input into the SAW **manualRegister** pipeline to modify registration record in IPR. Remember to run **imageTools ipr2img** again to acquire transformed images.

Running **manualRegister** requires the following files and information:

- count output gene expression matrix file (**.raw.gef**)
- An image processing output directory, including several **\*fov\_stitched\_transformed.tif** files, usually in **/path/to/output/04.register**
- **register** output IPR file (**.ipr**), supporting v0.2.1
- **StereoMap** manual registration output JSON file, recording manual operations (**.regist.json**)
- 🕒 Expected running time for 1 cm x 1 cm Stereo-seq Chip T image: ~3 min, Memory: 7 G

## 2.15.4.1 Arguments and Options

Table 2-19 manualRegister Arguments and Options

| Parameters | Function  |
|------------|---|
| <b>-i</b>  | (Required) register/rapidRegister output directory which includes a <stainType>_fov_stitched_transformed.tif or <stainType>_fov_stitched.tif file.  |
| <b>-c</b>  | (Required) register/rapidRegister output IPR file. manualRegister would overwrite registration parameters in the IPR to the manually adjusted parameters acquired from <b>StereoMap</b> .   |
| <b>-v</b>  | (Required) count output gene expression matrix GEF file.  |
| <b>-m</b>  | (Optional; default to "") The path to StereoMap output JSON file  |
| <b>-w</b>  | (Optional; default to False) False: negative degree when clockwise rotation. True: positive degree when clockwise rotation. Being valid when -m is missing.   |
| <b>-o</b>  | (Optional; default to 0 0) Offset in the x and y direction from the center of the fov_stitched_transformed.tif image. These two values are specified as "offsetX" and "offsetY" in the <b>StereoMap</b> output JSON file created after manual registration, and will be recorded as "OffsetX" and "OffsetY" in the IPR "Register" group. The offset x and offset y are separate by space.                         |
| <b>-f</b>  | (Optional; default to 0) Whether flip the image horizontally along the y-axis. This value is specified as "flip" in the <b>StereoMap</b> output JSON file created after manual registration. Valid options: 1 (flip), 0 (not flip). Flip information will be recorded as "Flip" in the IPR "Register" group.  |
| <b>-r</b>  | (Optional; default to 0) The manual rotation degree from the center of the fov_stitched_transformed.tif image. This value is specified as "rotate" in the <b>StereoMap</b> output JSON file created after manual registration, and will be recorded as "ManualRotation" in the IPR "Register" group.  |
| <b>-s</b>  | (Optional; default to 0 0) The manual scale in the x and y direction from the center off the fov_stitched_transformed.tif image. These two values are specified as "extrude_x" and "extrude_y" in the <b>StereoMap</b> output JSON file created after manual registration, and will be recorded as "ManualScaleX" and "ManualScaleY" in the IPR "Register" group. The scale x and scale y are separated by space. |
| <b>-p</b>  | (Required) Output directory.  |

## 2.15.4.2 Usage Examples



**Tip: get the inputs for manualRegister from StereoMap manual registration output JSON file:**

```

Option 1:
- "offsetX":{offsetX} of -o
- "offsetY":{offsetY} of -o
- "flip":{flip} of -f
- "rotate": {rotate} of -r
- "extrude_x": {extrude_x} of -s
- "extrude_y": {extrude_y} of -s
- "version":{isReversed} of -w,If this key is not present in the JSON, enter
"True". If it is present, enter "False". This parameter only takes effect when -m
is missing.

Option 2: Use StereoMap output JSON file only by -m.

```

**Scenario 1:** transform image based solely on manual operation

```

$ mkdir -p /path/to/output/manualregister
$ cp $(find /path/to/output/04.register -maxdepth 1 -name {SN}*.ipr | head -1)
/path/to/output/manualregister # we recommend to make a copy of IPR to pre-
vent overwrite original file
$ regIPR=$(find /path/to/output/manualregister -maxdepth 1 -name {SN}*.ipr |
head -1)

$ singularity exec SAW_v7.1.sif manualRegister \
  -i /path/to/output/04.register \
  -c ${regIPR} \
  -v /path/to/output/03.count/{SN}.raw.gef \
  -o {offsetX} {offsetY} \
  -f {flip} \
  -r {rotate} \
  -s {extrude_x} {extrude_y} \
  -w {isReversed} \
  -p /path/to/output/manualregister

```

**Scenario 2:** Use StereoMap output JSON file only

```

$ mkdir -p /path/to/output/manualregister
$ cp $(find /path/to/output/04.register -maxdepth 1 -name {SN}*.ipr | head -1)
/path/to/output/manualregister # we recommend to make a copy of IPR to pre-
vent overwrite original file
$ regIPR=$(find /path/to/output/manualregister -maxdepth 1 -name {SN}*.ipr |
head -1)

singularity exec SAW_v7.1.sif manualRegister \
  -i /path/to/output/04.register \
  -c ${regIPR} \
  -v /path/to/output/03.count/{SN}.raw.gef \
  -m /path/to/datetime.regist.json
  -p /path/to/output/manualregister

```

## 2.15.5 lasso

SAW lasso pipeline is performed to extract cell or spatial gene expression matrix(ces) of one or multiple manually delineated closed regions acquired from **StereoMap**.

Running **lasso** requires the following files and information:

- GEF file that includes **wholeExp** group or **cellCut** output cell bin GEF file (**.gef** or **.cellbin.gef**)
- **StereoMap** output coordinates list file (**.geojson**)
- 🕒 Running time and memory are highly dependent on the selected bin size and data volume.

### 2.15.5.1 Arguments and Options

Table 2-20 lasso Arguments and Options

| Parameters | Function  |
|------------|---|
| <b>-i</b>  | (Required) GEF file that includes wholeExp group or cellCut output cell bin GEF file (.gef or .cellbin.gef)               |
| <b>-m</b>  | (Required) StereoMap output coordinates list file (.geojson)  |
| <b>-n</b>  | (Required) The Stereo-seq Chip T serial number.   |
| <b>-o</b>  | (Required) Path to the directory where to store the lasso outputs. It has to be an existing path.                         |
| <b>-s</b>  | (Optional for square bin, default=1) GEM Bin size. Separate multiple bin size by comma, e.g. "-s 10,20".                  |
| <b>-O</b>  | (Optional, default=Transcriptomics) Omics name. Verify the value of Attribute "omics" in the input GEF/cell bin GEF file. |

### 2.15.5.2 Usage Examples



**Please Note!** Replace **{SN}** with your Stereo-seq Chip T serial number (SN, e.g. SS200000135TL\_D1), **{bin}** with the bin size you want (e.g. 1, 10, 200), and **/upload/path/{taskID}** with the true path and task ID.

Scenario 1: square bin lasso

```
$ mkdir -p /path/to/output/lasso
$ singularity exec SAW_v7.1.sif lasso \
  -i /path/to/output/03.count/{SN}.gef \
  -m /upload/path/{taskID}.lasso.geojson \
  -o /path/to/output/lasso \
  -s {bin} \
  -n {SN}
$ singularity exec SAW_v7.1.sif lasso \ # extract protein.gef subsets
  -i /path/to/output/04.calibration/{SN}.protein.gef \
  -m /upload/path/{taskID}.lasso.geojson \
  -o /path/to/output/lasso \
  -s {bin} -O Proteomics \
  -n {SN}
```

**Scenario 2: cell bin lasso**

```

$ mkdir -p /path/to/output/lasso

$ singularity exec SAW_v7.1.sif lasso \
  -i /path/to/output/051.tissuecut/{SN}.cellbin.gef \
  -m /upload/path/{taskID}.lasso.geojson \
  -o /path/to/output/lasso \
  -n {SN} # -O Proteomics is required in extracting {SN}.protein.cellbin.gef

# do cell cluster for cellbin.gef and can show clusters in StereoMap
$ singularity exec SAW_v7.1.sif lasso \
  -i /path/to/output<label>/{SN}.<label>.label.cellbin.gef \
  -o /path/to/output<label>/{SN}.<label>.label.cell.cluster.h5ad

# pre-compute rendering data and can show cellbin.gef expression heatmap in
StereoMap
$ singularity exec SAW_v7.1.sif lasso \
  -i /path/to/output<label>/{SN}.<label>.label.cell.cluster.h5ad \
  -o /path/to/<label>

```

### 2.15.5.3 Outputs

Square bin lasso output files are:

```

$ tree /path/to/output/lasso
/path/to/output/lasso
├── <label>
│   ├── SN.<label>.label.gef
│   ├── segmentation
│   │   ├── SN.lasso.<binList[0]>.<label>.gem.gz
│   │   ├── ...
│   │   └── SN.lasso.<binList[m]>.<label>.gem.gz
│   └── SN.lasso.<label>.mask.tif

```

Cellbin lasso output files are:

```

$ tree /path/to/output/lasso
/path/to/output/lasso
├── <label>
│   └── SN.<label>.label.cellbin.gef

```

## 2.15.6 cellChunk

cellChunk is performed to generate precomputed rendering data for StereoMap. This information will be recorded in /codedCellBlock in cell bin GEF file.

Running cellChunk requires the following file:

- cell bin GEF file (**cellbin.gef**) after cellCluster.

### 2.15.6.1 Arguments and Options

Table 2-21 cellChunk Arguments and Options

| Parameters | Function  |
|------------|---|
| <b>-i</b>  | (Required) cell bin GEF file (.cellbin.gef or .cellbin.gef ) after <b>cellCluster</b> . |
| <b>-o</b>  | (Required) Path to the directory where to store outputs. It has to be an existing path. |

### 2.15.6.2 Usage Examples

```

$ mkdir -p /path/to/output/lasso

$ singularity exec SAW_v7.1.sif cellChunk \
  -i /path/to/output/051.cellcut/{SN}.adjusted.cellbin.gef \
  -o /path/to/051.cellcut

```

## 2.15.7 MIDFilter

MIDFilter is performed to manually filter spatial expression matrixes by MID range acquired from StereoMap.

Running MIDFilter requires the following file:

- GEF file that includes wholeExp group (**.gef**)
- StereoMap output coordinates list file (**.MIDFilter.json**)

### 2.15.7.1 Arguments and Options

Table 2-22 MIDFilter Arguments and Options

| Parameters | Function   |
|------------|--|
| <b>-i</b>  | (Required) GEF file that includes wholeExp group (.gef)                  |
| <b>-m</b>  | (Required) StereoMap output manual MID filtration file (.MIDFilter.json) |
| <b>-o</b>  | (Required) Output GEF file.  |

### 2.15.7.2 Usage Examples

```
$ singularity exec SAW_v7.1.sif MIDFilter \  
  -i /path/to/output/04.calibration/{SN}.protein.gef \  
  -m /path/to/datetime.FilterMID.json \  
  -o /path/to/output/{SN}.protein.filter.gef
```



# CHAPTER 3

## SAW PIPELINES & ARGUMENTS

–

## PROTEOMICS & TRANSCRIPTOMICS

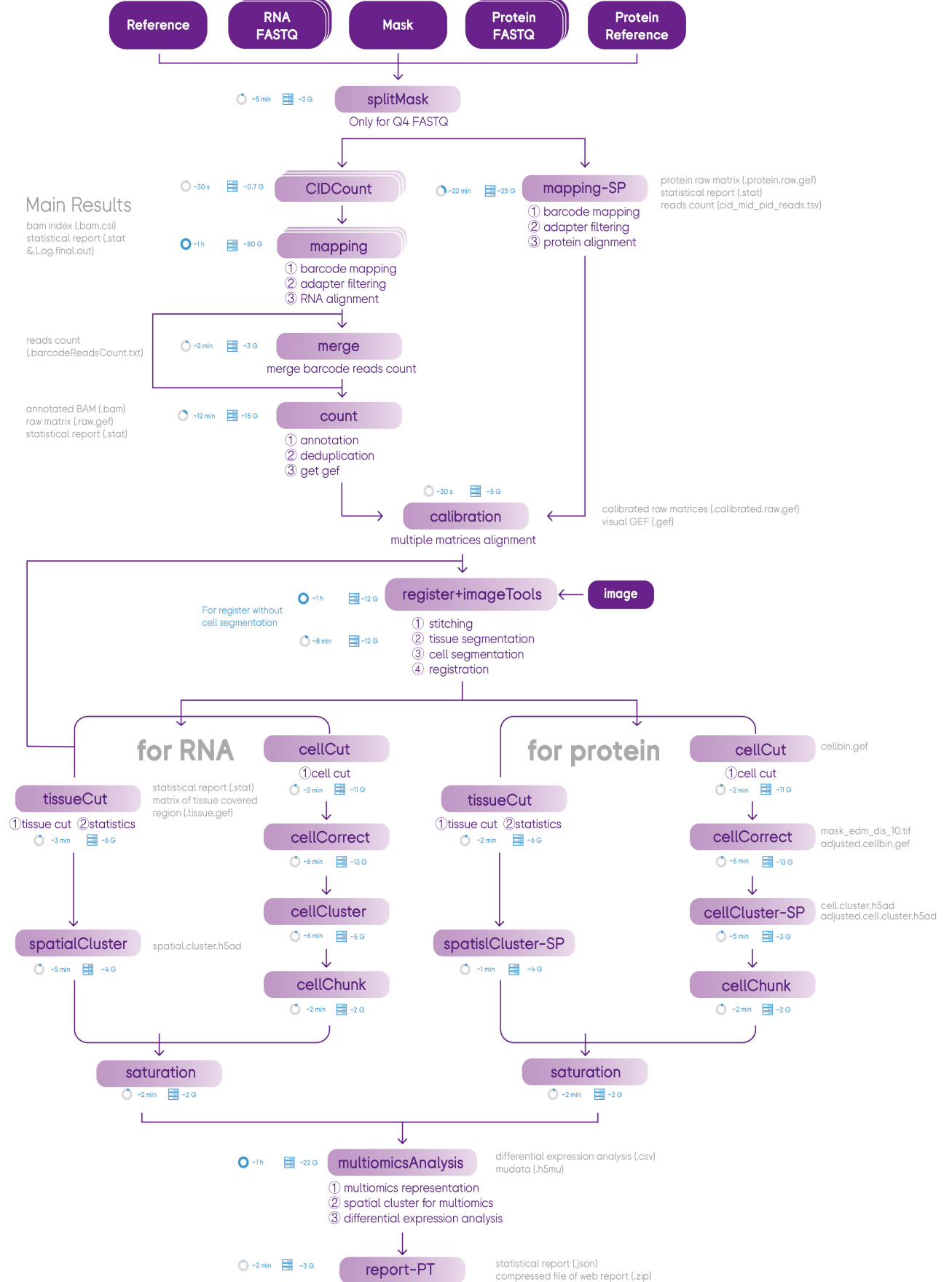
# WORKFLOW

RNA FASTQ: 1G  
 Protein FASTQ: 0.5G-1G  
 Protein Reference:  
 119 proteins + 9 isoforms

64 bit CentOS/RedHat 7.8  
 64 bit Ubuntu 20.04

Minimum requirements:  
 8 cores 128 G 1 TB

Higher requirements:  
 24 cores 256 G 1 TB



## 3.1 Shortcuts of STOmics-RNA reads mapping

### 3.1.1 SplitMask (Required for Q4 FASTQ input case)

Split Stereo-seq Chip T mask file according to the Q4 FASTQ CID. The split count is directly related to the number of split FASTQs while writing FASTQ out from sequencer.

Running `splitMask` requires the following files:

- Stereo-seq Chip T mask file (**.h5**)

 Please check [2.1 splitMask](#) to learn more this pipeline.

#### 3.1.1.1 Usage Example

```

$ mkdir /path/to/output/00.splitmask
$ singularity exec SAW_v7.1.sif splitMask \
  /path/to/data/{SN}.barcodeToPos.h5 \ ## Mask path
  /path/to/output/00.splitmask \ ## output directory
  8 \ ## threads
  16 \ ## split count
  2_25 ## CID position

```

### 3.1.2 CIDCount

Compute the number of CIDs in the Stereo-seq Chip T mask file and roughly estimate memory used in mapping.

Running `CIDCount` requires the following files:

- Stereo-seq Chip T mask file (**.h5**)

 Please check [2.2 CIDCount](#) to learn more this pipeline.

#### 3.1.2.1 Usage Example

For Q40 FASTQ input case, run `CIDCount` as below.

```

$ singularity exec SAW_v7.1.sif CIDCount \
  -i /path/to/data/{SN}.barcodeToPos.h5 \ ## Stereo-seq Chip T mask file path
  -g {genomeSize} ## genome file size in GB, can be acquired by "ls -l
  --block-size=GB ${Genome file of the species after STAR indexing}"


```

### 3.1.3 mapping

Correspond in situ captured STOmics-RNA sequenced reads recorded in FASTQ(3,4) files by Stereo-seq with their spatial information. It also aligns reads to the reference genome and generates coordination sorted BAM files.

Running **mapping** requires the following files:

- Stereo-seq sequenced reads FASTQ files (**.fq.gz**)
- Stereo-seq Chip T mask file (**.h5**)
- Indexed reference genome
- bcPara file (**.bcPara**)

 Please check [2.4 mapping](#) to learn more about Q40 multiple pairs FASTQ and Q4 FASTQ input cases.

#### 3.1.3.1 Usage Example

**Q40 scenario 1:** Prepare `{lane}.bcPara` file for Q40 one pair FASTQ as **mapping** input:

```
$ mkdir /path/to/output/01T.mapping
$ vim /path/to/output/01T.mapping/{lane}.bcPara
in=/path/to/data/{SN}.barcodeToPos.h5
in1=/path/to/data/{lane}_read_1.fq.gz
in2=/path/to/data/{lane}_read_2.fq.gz
barcodeReadsCount=/path/to/ouputut/01T.mapping/{lane}.barcodeReadsCount.txt
barcodeStart=0
barcodeLen=25
umiStart=25
umiLen=10
mismatch=1
bcNum=645784920 ## Input the first line from output of CIDCount
polyAnum=15
mismatchInPolyA=2
```

Run **mapping** for Q40 input:

```
$ singularity exec SAW_v7.1.sif mapping \
  --outSAMattributes spatial \
  --outSAMtype BAM SortedByCoordinate \
  --genomeDir /path/to/genomeDir \
  --runThreadN 8 \
  --outFileNamePrefix /path/to/output/01T.mapping/{lane}. \
  --sysShell /bin/bash \
  --stParaFile /path/to/output/01T.mapping/{lane}.bcPara \
  --readNameSeparator "\" \" \
  --limitBAMsortRAM 38582880124 \
  --limitOutSJcollapsed 10000000 \
  --limitIObufferSize=280000000 \
  --outBAMsortingBinsN 50 \
  > /path/to/output/01T.mapping/{lane}.run.log
```

### 3.1.4 merge

Combine the results of mapping.

Running **merge** requires the following file:

- **mapping** output mapped CID list files (**.txt**)

 Please check [2.4 merge](#) to learn more this pipeline.

#### 3.1.4.1 Usage Example

```

$ mkdir /path/to/multi_lane_output/02T.merge
$ singularity exec SAW_v7.1.sif merge \
    /path/to/data/{SN}.barcodeToPos.h5 \
    /path/to/multi_lane_output/01T.mapping/{lane1}.barcodeReadsCount.txt,
/path/to/
multi_lane_output/01T.mapping/{lane2}.barcodeReadsCount.txt \ ## change {lane} to
{index} or {idx} for Q4 and change /path/to/multi_lane_output/ to /path/to/output/
for SE single lane scenario
/path/to/multi_lane_output/02T.merge/{SN}.barcodeReadsCount.txt

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

### 3.1.5 count

Reads BAM files generated from mapping to perform gene annotation, de-duplication, and gene expression analysis on the aligned reads.

Running **count** requires the following files:

- **mapping** output BAM file (**.bam**)
- Reference genome annotation GFF/GTF<sup>9,10</sup> file (**.gff / .gtf**)

 Please check [2.5 count](#) to learn more this pipeline.

### 3.1.5.1 Usage Example

For one pair of FASTQ files:

```
$ mkdir -p /path/to/output/03T.count
$ geneExp=/path/to/output/03T.count/{SN}.raw.gcf
$ saturationSamplingFile=/path/to/output/03T.count/{SN}_raw_barcode_gene_exp.txt
$ singularity exec SAW_v7.1.sif count \
    -i /path/to/output/01T.mapping/{lane}.Aligned.sortedByCoord.out.bam \
    -o /path/to/output/03T.count/
[*] {SN}.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam \
    -a /path/to/reference/genes.gtf \
    -s /path/to/output/03T.count/
[*] {SN}.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam.summary.stat \
    -e ${geneExp} \
    --umi_len 10 \
    --sat_file ${saturationSamplingFile} \
    --sn {SN} \
    --umi_on \
    --save_lq \
    --save_dup \
    -c 24
```

## 3.2 mapping-SP

Each Stereo-seq sequenced read contains a CID sequence which is used as a key to spatially map the read back to its original location on the tissue section. **mapping-SP** matches CID of the original sequencing reads stored in the FASTQ file with the records of CID-coordinates key-value pairs saved in the Stereo-seq Chip T mask file (allow 1 mismatch). Coordinate information for reads that CID could be paired with, based on the records of the mask file, will be added. Reads that get the coordinate annotations are Valid CID Reads (**Valid CID Reads**). After discarding reads with unqualified MID reads which do not satisfy further analysis, the filtered **Valid CID Reads** are mapped to the protein database, and output protein expression matrix (GEF & GEM) and summary report.

Running **mapping-SP** requires the following files:

- Stereo-seq sequenced reads FASTQ files (**.fq.gz**)
- Stereo-seq Chip T mask file (**.h5**)
- Protein database file
- 🕒 Expected running time for ~1G reads: ~10 min, Memory: ~25G



Please confirm the protein database file (used in `--reference`) only records the actual input proteins. You can get protein database in [SAW Github](#).

### 3.2.1 Arguments and Options

Table 3-1 **mapping-SP Arguments and Options**

| Parameter                  | Function  |
|----------------------------|---|
| <code>--mask</code>        | (Required) Stereo-seq Chip T mask file path (.h5 or .bin).  |
| <code>--fastq</code>       | (Required) Q40 FASTQ file of read1 list or Q4 FASTQ list.   |
| <code>--fastq2</code>      | (Optional) Q40 FASTQ file of read2 list.  |
| <code>--reference</code>   | (Required) PID database file.   |
| <code>--cidStart</code>    | (Required; defaults to 0) CID start position.   |
| <code>--cidLen</code>      | (Required; defaults to 25) CID length. Set to 25 for Q40 FASTQ, 24 for Q4 FASTQ.  |
| <code>--cidMismatch</code> | (Required) Genome file size in GB.  |
| <code>--cidMismatch</code> | (Required; defaults to 0) Max CID mismatch tolerant. Usually set to 1 in mapping-SP. If set to 0, then the reads that contain an N will be deleted. |
| <code>--midStart</code>    | (Required; defaults to 25) MID start position.  |
| <code>--midLen</code>      | (Required; defaults to 10) MID length.  |
| <code>--pidStart</code>    | (Required; default to 0) PID start position.  |
| <code>--pidLen</code>      | (Required; default to 15) PID length.   |
| <code>--pidMismatch</code> | (Required; defaults to 0) Max PID mismatch tolerant. Usually set to 1 in mapping-SP. If set to 0, then the reads that contain an N will be deleted. |
| <code>--output, -o</code>  | (Required) Output directory.  |

| Parameter                   | Function   |
|-----------------------------|--|
| <code>--sn</code>           | (Required) Stereo-seq Chip T serial number.  |
| <code>--thread</code>       | (Required, default=1) Set the number of threads to be used. Usually set to 8 or higher.          |
| <code>--proteinList</code>  | (Optional) The protein-list file path (.txt), that records added proteins during the experiment. |
| <code>--limitSortRAM</code> | (Optional) Max RAM for sorting.  |


### 3.2.2 Usage Example

Preparing **mapping-SP** input file `{SN}.protein.database.txt`. The protein database records the antibody has been used in the sample, which includes three columns: PIDindex, PIDsequence and PIDname. "PIDName" only accepts letters [a-zA-Z], digits [0-9] and symbols ["(", ")", "-", "\_"]. Each column is separated by **tab**. Here is an example:

```

PIDIndex      PIDSequence      PIDName
0             GCCGGACGACATTAA  Isotype_Ms_IgG1k
1             CTCCTACCTAAACTG  Isotype_Ms_IgG2ak
2             ATATGTATCACGCGA  Isotype_Ms_IgG2bk
3             GATTCTTGACGACCT  Isotype_Rat_IgG2bk
4             ATCAGATGCCCTCAT  Isotype_Rat_IgG1k
5             GGGAGCGATTCAACT  Isotype_Rat_IgG1r
6             AAGTCAGGTTGTTT   Isotype_Rat_IgG2ak
7             TCCAGGCTAGTCATT  Isotype_Rat_IgG2ck
8             CCTGTCATTAAGACT  Isotype_Ham_IgG
9             ATGTACCCGGTGTGT  Isotype_Rat_IgMk

```

 **Please Note!** Replace `{SN}` with your Stereo-seq Chip T serial number (SN, e.g. SS200000135TL\_D1 or A00135D1), and `{lane}` with the FASTQ lane name prefix (e.g. E100026571\_L01). The same applies to all examples.

**Q40 scenario 1:** one pair of FASTQ as **mapping-SP** input:

```

# one pairs of FASTQ
$ mkdir /path/to/output/01P.mapping
$ singularity exec SAW_v7.1.sif mapping-SP \
  --thread 8 \
  --cidStart 0 --cidLen 25 --cidMismatch 1 \
  --midStart 25 --midLen 10 \
  --pidStart 21 --pidLen 15 --pidMismatch 1 \
  --mask /path/to/data/{SN}.barcodeToPos.h5 \
  --fastq /path/to/data/{lane}_read_1.fq.gz \
  --fastq2 /path/to/data/{lane}_read_2.fq.gz \
  --reference /path/to/data/{SN}.protein.database.txt \ ## you can use protein
panel in SAW Github directly
--output /path/to/output/01P.mapping \
--sn {SN}

```



**Q40 scenario 2:** multiple pairs of FASTQ as **mapping-SP** input:

Prepare `adtFq1.list` and `adtFq2.list` file:

```

$ cat /path/to/output/01P.mapping/adtFq1.list # one FASTQ file in a row
/path/to/data/lane_1_read_1.fq.gz
/path/to/data/lane_2_read_1.fq.gz

$ cat /path/to/output/01P.mapping/adtFq2.list # sorted by adtFq1.list
/path/to/data/lane_1_read_2.fq.gz
/path/to/data/lane_2_read_2.fq.gz

```

Multiple pairs of Q40 FASTQ as input:

```

$ mkdir /path/to/output/01P.mapping
$ singularity exec SAW_v7.1.sif mapping-SP \
  --thread 8 \
  --cidStart 0 --cidLen 25 --cidMismatch 1 \
  --midStart 25 --midLen 10 \
  --pidStart 21 --pidLen 15 --pidMismatch 1 \
  --mask /path/to/data/{SN}.barcodeToPos.h5 \
  --fastq /path/to/output/01P.mapping/adtFq1.list \
  --fastq2 /path/to/output/01P.mapping/adtFq2.list \
  --reference /path/to/data/{SN}.proteinDatabase.txt \ ## you can use protein
  panel in SAW Github directly
  --output /path/to/output/01P.mapping \
  --sn {SN}

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

**Q4 scenario :** prepare **mapping-SP** input file `adtFq1.list` with Q4 FASTQ input:

SE FASTQ name

`/path/to/data/E100026571_L01/barcode_2/E100026571_L01_2_16.fq.gz`

lane

barcode

lane

barcode

split index

```

$ cat /path/to/output/01P.mapping/adtFq1.list
/path/to/data/lane_1_16.fq.gz
/path/to/data/lane_2_16.fq.gz

```

Run mapping-SP pipeline for Q4 input:

```
•••
$ mkdir /path/to/output/01P.mapping
$ find /path/to/output/00.splitmask/splitBin/*.{SN}.barcodeToPos.bin>splitMask.txt
$ singularity exec SAW_v7.1.sif mapping-SP \
  --thread 8 \
  --cidStart 0 --cidLen 24 --cidMismatch 1 \
  --midStart 25 --midLen 10 \
  --pidStart 21 --pidLen 15 --pidMismatch 1 \
  --mask /path/to/output/01P.mapping/splitMask.txt \
  --fastq /path/to/output/01P.mapping/adtFq1.list \
  --reference /path/to/data/{SN}.protein.database.txt \
  --output /path/to/output/01P.mapping \
  --sn {SN}
```

### 3.2.3 Outputs

Q40 & Q4 scenarios output files are organized as below:

```
•••
$ tree /path/to/output/01P.mapping/
/path/to/output/01P.mapping/
├── SN_cid_pid_mid_reads.tsv
├── SN_map.stat
├── SN.protein.gem.gz
├── SN.protein.raw.gef
└── SN_valid_cid_reads.tsv
```

### 3.3 calibration

SAW **calibration** transforms two expression matrices into the same size. The output shape of two matrices is the external-rectangle of two original matrices.

Running **calibration** requires the following files:

- **count** output transcriptomics gene expression matrix file (**.raw.gef**)
- **mapping-SP** output proteomics expression matrix file (**.protein.raw.gef**)

🕒 Expected running time for ~1G reads: ~0.5 min, Memory: ~2G

#### 3.3.1 Arguments and Options

Table 3-2 **calibration** Arguments and Options

| Parameter | Function  |
|-----------|---|
| <b>-i</b> | (Required) Path to input GEF files, seperated by comma.   |
| <b>-o</b> | (Required) Path to output calibrated GEF files, corresponding to input GEF and separated by comma.                    |
| <b>-0</b> | (Required) Omics name, corresponding to input GEF and separated by comma. Valid options: Transcriptomics, Proteomics. |

#### 3.3.2 Usage Example

```

$ singularity exec SAW_v7.1.sif calibration \
  -i /path/to/output/03T.count/{SN}.raw.gef,/path/to/output/01P.mapping/
  {SN}.protein.raw.gef \
  -o /path/to/output/04.calibration/{SN}.calibrated.raw.gef,
  /path/to/output/04.calibration/{SN}.protein.calibrated.raw.gef \
  -0 Transcriptomics,Proteomics

```

\* Please be noted that these two lines belong to the same line of command.

#### 3.3.3 Outputs

```

$ tree /path/to/output/04.calibration/
/path/to/output/04.calibration/
├── SN.calibrated.raw.gef
└── SN.protein.calibrated.raw.gef

```

## 3.4 Shortcuts of register & imageTools

### 3.4.1 register

SAW **register** pipeline aligns the microscopic tissue staining images with the plot of gene expression matrix generated by **calibration** based on the track lines on the chip while establishing the mapping relationship between images and spatial gene expression distribution. Staining images include:

Running **register** requires the following files:

- **calibration** output gene expression matrix file (**.calibrated.raw.gcf**)
- The microscopic tissue staining image file (**.tar.gz**) processed by **ImageStudio**. **register** is compatible with QC outputs from **ImageStudio v3**.
- **ImageStudio** Image process record file (**.ipr**)

 Please check [2.6 register](#) to learn more scenarios.

#### 3.4.1.1 Usage Example

**Scenario 1:** Process images from ImageStudio output data and gene expression matrix. Perform stitching, tissue segmentation, cell segmentation, and registration with gene expression matrix.

```

$ image=/path/to/data/image
$ image4register=$(find ${image} -maxdepth 1 -name {SN}*.tar.gz | head -1)$
imageQC=$(find ${image} -maxdepth 1 -name {SN}*.ipr | head -1)
$ threads=<threads num>

$ mkdir -p /path/to/output/05.register
$ singularity exec SAW_v7.1.sif register \
  -i ${image4register} \
  -c ${imageQC} \
  -v /path/to/output/04.calibration/{SN}.calibrated.raw.gcf \
  -o /path/to/output/05.register \
  -w True --core ${threads} ## do cell segmentation

```

### 3.4.2 imageTools

SAW **imageTools ipr2img** outputs TIFF images from IPR such as pre-registered ssDNA stitched image, tissue segmentation and cell segmentation mask TIFFs, as well as registered three types of images.

Running **imageTools ipr2img** requires the following files:

- ImageStudio processed microscopic tissue staining image file (**.tar.gz**)
- **register** processed Image process record file (**.ipr**), supporting v0.2.1

 Please check [2.7 imageTools](#) to learn more about **imageTools ipr2img**.

### 3.4.2.1 Usage Example

```
...
$ image=/path/to/data/image
$ image4register=$(find ${image} -maxdepth 1 -name {SN}*.tar.gz | head -1)
$ imageIPR=$(find /path/to/output/05.register -maxdepth 1 -name {SN}*.ipr | head -1)
## has to be a processed IPR

$ singularity exec SAW_v7.1.sif imageTools ipr2img \
  -i ${image4register} \
  -c ${imageIPR} \
  -d tissue cell \ ## output both tissue and cell segmentation mask TIFF
  -r True \
  -o /path/to/output/05.register
```

### 3.5 tissueCut

SAW **tissueCut** pipeline can delineate and extract the tissue coverage area based on the aligned image generated from **register** and **imageTools** or from the plot of gene expression matrix (if microscopic tissue staining images are not available). **tissueCut** outputs expression data in GEF format.



If the output of **tissueCut** doesn't match the morphology of the tissue, users could handle this issue with the help of **ImageStudio**, **StereoMap** or community developed tools, such as **Stereopy**<sup>11</sup>. If the **tissueCut** was run with an image, users could manually redo tissue segmentation for the image via **ImageStudio** and run **register**, **imageTools** and **tissueCut** again. Otherwise, manually delineate tissue coverage region from the gene expression distribution plot via **StereoMap** and then put the result into the SAW-lasso pipeline (2.15.6 lasso).

Running **tissueCut** for Proteomics mode requires the following files:

- **mapping-SP** output mapped CID list file (**SN\_valid\_cid\_read.tsv**)
- **calibration** output protein expression matrix file (**.protein.calibrated.raw.gef**)
- **imageTools ipr2img** output tissue segmentation mask TIFF file (**.tif, optional**)

Running **tissueCut** for Transcriptomics mode requires the following files:

- Mapped CID list file (**.barcodeReadsCount.txt**)
- **calibration** output gene expression matrix file (**.calibrated.raw.gef**)
- **imageTools ipr2img** output tissue segmentation mask TIFF file (**.tif, optional**)

⌚ Expected running time for ~1G reads: ~6 min, Memory: ~6 G

#### 3.5.1 Arguments and Options

Table 3-3 **tissueCut** Arguments and Options

| Parameter          | Function   |
|--------------------|--|
| <b>--dnbfile</b>   | (Optional) Mapped CID list file with reads counts for each CID. Input merged mapped CID list file if more than one list file was generated in <b>mapping</b> . |
| <b>-i</b>          | (Required) <b>count</b> output gene expression matrix file.  |
| <b>-o</b>          | (Required) Path to the directory where to store the <b>tissueCut</b> outputs.  |
| <b>-s</b>          | (Optional) Path to the <b>imageTools ipr2img</b> output tissue segmentation mask file. Only valid when <b>register</b> has been performed.                     |
| <b>--sn</b>        | (Required) Stereo-seq Chip T serial number (SN).   |
| <b>--omics, -0</b> | (Required; default to Transcriptomics) String that specifies the omics.  |
| <b>-d</b>          | (Required) Set to generate required plots for report.  |
| <b>-t</b>          | (Optional) Number of threads used in numpy (recommended to fill 8 here).   |
| <b>-b</b>          | (Optional; default to 1,20,50,100,200) Set to specify bin sizes, separated by comma.   |
| <b>-l</b>          | (Optional) Set to specify the output label name.   |

### 3.5.2 Usage Example

Run `tissueCut` if `register` aligned microscopic staining image is provided:

```

...
$ nucleusLayer=$(find /path/to/output/05.register -maxdepth 1 -name *fov_stitched_
transformed.tif -exec sh -c 'for f do basename -- "$f" _fov_stitched_transformed.
tif;done' sh {} + | grep -v IF)
$ tissueMaskFile=$(find /path/to/output/05.register -maxdepth 1 -name ${nucle-
usLayer}*_tissue_cut.tif

$ mkdir -p /path/to/output/06P.tissuecut
$ singularity exec SAW_v7.1.sif tissueCut \
  --dnbfile /path/to/output/01P.mapping-SP/{SN}_valid_cid_read.tsv \
  -i /path/to/output/04.calibration/{SN}.protein.calibrated.raw.gef \
  -o /path/to/output/06P.tissuecut \
  -s ${tissueMaskFile} \
  --sn {SN} -O Proteomics -d

$ mkdir -p /path/to/output/06T.tissuecut
$ singularity exec SAW_v7.1.sif tissueCut \
  --dnbfile /path/to/output/02.merge/{SN}.merge.barcodeReadsCount.txt \
  -i /path/to/output/04.calibration/{SN}.calibrated.raw.gef \
  -o /path/to/output/06T.tissuecut \
  -s ${tissueMaskFile} \
  --sn {SN} -O Transcriptomics -d

```

Run `tissueCut` if image is not provided:

```

...
$ mkdir -p /path/to/output/06P.tissuecut
$ singularity exec SAW_v7.1.sif tissueCut \
  --dnbfile /path/to/output/00P.mapping/{SN}_valid_cid_read.tsv \
  -i /path/to/output/04.calibration/{SN}.protein.calibrated.raw.gef \
  -o /path/to/output/06P.tissuecut \
  --sn {SN} -O Proteomics -d

$ mkdir -p /path/to/output/06T.tissuecut
$ singularity exec SAW_v7.1.sif tissueCut \
  --dnbfile /path/to/output/02.merge/{SN}.merge.barcodeReadsCount.txt \
  -i /path/to/output/04.calibration/{SN}.calibrated.raw.gef \
  -o /path/to/output/06T.tissuecut \
  --sn {SN} -O Transcriptomics -d

```

### 3.5.3 Outputs

**tissueCut** output files:

Image is provided:

- Generate tissue GEF based on the corresponding tissue mask image.

```
$ tree /path/to/output/06P.tissuecut
/path/to/output/06P.tissuecut
├── SN.protein.tissue.gef
├── tissuecut.stat
└── tissue_fig
    ├── scatter_100x100_MID_gene_counts.png
    ├── scatter_150x150_MID_gene_counts.png
    ├── scatter_200x200_MID_gene_counts.png
    ├── scatter_20x20_MID_gene_counts.png
    ├── scatter_50x50_MID_gene_counts.png
    ├── statistic_100x100_DNB.png
    ├── statistic_100x100_gene.png
    ├── statistic_100x100_MID.png
    ├── statistic_150x150_DNB.png
    ├── statistic_150x150_gene.png
    ├── statistic_150x150_MID.png
    ├── statistic_200x200_DNB.png
    ├── statistic_200x200_gene.png
    ├── statistic_200x200_MID.png
    ├── statistic_20x20_DNB.png
    ├── statistic_20x20_gene.png
    ├── statistic_20x20_MID.png
    ├── statistic_50x50_DNB.png
    ├── statistic_50x50_gene.png
    ├── statistic_50x50_MID.png
    ├── violin_100x100_gene.png
    ├── violin_100x100_MID.png
    ├── violin_150x150_gene.png
    ├── violin_150x150_MID.png
    ├── violin_200x200_gene.png
    ├── violin_200x200_MID.png
    ├── violin_20x20_gene.png
    ├── violin_20x20_MID.png
    ├── violin_50x50_gene.png
    └── violin_50x50_MID.png
```

```
$ tree /path/to/output/06T.tissuecut
/path/to/output/06T.tissuecut
├── SN.tissue.gef
├── tissuecut.stat
└── tissue_fig
    ├── scatter_100x100_MID_gene_counts.png
    ├── scatter_150x150_MID_gene_counts.png
    ├── scatter_200x200_MID_gene_counts.png
    ├── scatter_20x20_MID_gene_counts.png
    ├── scatter_50x50_MID_gene_counts.png
    ├── statistic_100x100_MID_gene_DNB.png
    ├── statistic_150x150_MID_gene_DNB.png
    ├── statistic_200x200_MID_gene_DNB.png
    ├── statistic_20x20_MID_gene_DNB.png
    ├── statistic_50x50_MID_gene_DNB.png
    ├── violin_100x100_MID_gene.png
    ├── violin_150x150_MID_gene.png
    ├── violin_200x200_MID_gene.png
    ├── violin_20x20_MID_gene.png
    └── violin_50x50_MID_gene.png
```



Image is not provided:

```

$ tree /path/to/output/06P.tissuecut
/path/to/output/06P.tissuecut
├── 100X100_contour_image.png
├── bin1_img.tif
## bin1 expression distribution TIFF file
├── bin1_img_tissue_cut.tif
## tissue mask acquried from
## bin1 expression distribution plot
├── SN.protein.tissue.gef
├── tissuecut.stat
└── tissue_fig
    ├── scatter_100x100_MID_gene_counts.png
    ├── scatter_150x150_MID_gene_counts.png
    ├── scatter_200x200_MID_gene_counts.png
    ├── scatter_20x20_MID_gene_counts.png
    ├── scatter_50x50_MID_gene_counts.png
    ├── statistic_100x100_DNB.png
    ├── statistic_100x100_gene.png
    ├── statistic_100x100_MID.png
    ├── statistic_150x150_DNB.png
    ├── statistic_150x150_gene.png
    ├── statistic_150x150_MID.png
    ├── statistic_200x200_DNB.png
    ├── statistic_200x200_gene.png
    ├── statistic_200x200_MID.png
    ├── statistic_20x20_DNB.png
    ├── statistic_20x20_gene.png
    ├── statistic_20x20_MID.png
    ├── statistic_50x50_DNB.png
    ├── statistic_50x50_gene.png
    ├── statistic_50x50_MID.png
    ├── violin_100x100_gene.png
    ├── violin_100x100_MID.png
    ├── violin_150x150_gene.png
    ├── violin_150x150_MID.png
    ├── violin_200x200_gene.png
    ├── violin_200x200_MID.png
    ├── violin_20x20_gene.png
    ├── violin_20x20_MID.png
    ├── violin_50x50_gene.png
    └── violin_50x50_MID.png

```

```

$ tree /path/to/output/06T.tissuecut
/path/to/output/06T.tissuecut
├── 100X100_contour_image.png
├── bin1_img.tif
## bin1 expression distribution TIFF file
├── bin1_img_tissue_cut.tif
## tissue mask acquried from
## bin1 expression distribution plot
├── SN.tissue.gef
├── tissuecut.stat
└── tissue_fig
    ├── scatter_100x100_MID_gene_counts.png
    ├── scatter_150x150_MID_gene_counts.png
    ├── scatter_200x200_MID_gene_counts.png
    ├── scatter_20x20_MID_gene_counts.png
    ├── scatter_50x50_MID_gene_counts.png
    ├── statistic_100x100_MID_gene_DNB.png
    ├── statistic_150x150_MID_gene_DNB.png
    ├── statistic_200x200_MID_gene_DNB.png
    ├── statistic_20x20_MID_gene_DNB.png
    ├── statistic_50x50_MID_gene_DNB.png
    ├── violin_100x100_MID_gene.png
    ├── violin_150x150_MID_gene.png
    ├── violin_200x200_MID_gene.png
    ├── violin_20x20_MID_gene.png
    └── violin_50x50_MID_gene.png

```

### 3.6 spatialCluster-SP & spatialCluster

SAW **spatialCluster-SP** & **spatialCluster** pipeline performs clustering analysis for spots using Stereopy. The clustering procedure includes 4 main steps: 1) preprocess expression data from the tissue coverage region (normalize, logarithmize, identify highly variable genes, and scale each gene/protein), 2) reduce the dimensionality of the data by running PCA, 3) compute the neighborhood graph and embed neighborhood graph using UMAP, 4) clustering by Leiden algorithm.

Running **spatialCluster-SP** requires the following files:

- **tissueCut** output protein expression file for the tissue-covered region (**.protein.tissue.gef**)

Running **spatialCluster** requires the following files:

- **tissueCut** output gene expression file for the tissue-covered region (**.tissue.gef**)

🕒 Expected running time for ~1G reads: ~1 min, Memory: ~5 G

#### 3.6.1 Arguments and Options

Table 3-4 **spatialCluster** Arguments and Options

| Parameter | Function   |
|-----------|--|
| <b>-i</b> | (Required) <b>tissueCut</b> output GEF file for the tissue coverage area.  |
| <b>-o</b> | (Required) Output path for the clustering result in H5AD format.   |
| <b>-s</b> | (Required; default to 50) Bin size.  |
| <b>-r</b> | (Required; default to 1.0) Leiden resolution which controls the coarseness of the clustering. Higher values lead to more clusters. |

#### 3.6.2 Usage Example

```

$ mkdir -p /path/to/output/07P.spatialcluster
$ singularity exec SAW_v7.1.sif spatialCluster \
-i /path/to/output/06P.tissuecut/{SN}.protein.tissue.gef \
-s 200 \
-o /path/to/output/07P.spatialcluster/{SN}_bin200_0.1.protein.spatial.cluster.h5ad
## Default Leiden resolution for proteomics is 0.1

$ mkdir -p /path/to/output/07T.spatialcluster
$ singularity exec SAW_v7.1.sif spatialCluster \
-i /path/to/output/06T.tissuecut/{SN}.tissue.gef \
-r 1.0 \
-s 200 \
-o /path/to/output/07T.spatialcluster/{SN}_bin200_1.0.spatial.cluster.h5ad

```

#### 3.6.3 Outputs

**spatialCluster-SP** output files are:

```

$ tree /path/to/output/07P.spatialcluster
/path/to/output/07P.spatialcluster
├── SN_bin200_0.1.protein.spatial.cluster.h5ad

$ tree /path/to/output/07T.spatialcluster
/path/to/output/07T.spatialcluster
├── SN_bin200_1.0.spatial.cluster.h5ad

```

## 3.7 cellCut

SAW **cellCut** pipeline runs to extract expression matrix of cell nucleus based on the aligned image generated from **register** and **imageTools**. **cellCut** outputs expression data in cell bin GEF format. Run **cellCut** if cell bin results are desired.

Running **cellCut** requires the following files:

- **calibration** output expression matrix file (**.calibrated.raw.gef**)
- **register** and **imageTools** output cell segmentation mask TIFF file (**.tif**)

🕒 Expected running time for ~1G reads: ~2 min, Memory: ~10 G

### 3.7.1 Arguments and Options

Table 3-5 **cellCut** Arguments and Options

| Commands                    | Parameters   | Function  |
|-----------------------------|--|---|
| cgef                        | <b>-i, --input-file</b>                                      | (Required) Input GEF file.  |
|                             | <b>-m, --mask-file</b>                                       | (Required) Input cell segmentation mask file.                                 |
|                             | <b>-o, --output-file</b>                                     | (Required) Output cell bin GEF file.  |
|                             | <b>-b, --block</b>   | (Optional; default to 256,256) Block size.                                    |
|                             | <b>-r, --rand-celltype</b>                                   | (Optional; default to 0) Number of random cell type.                          |
|                             | <b>-t, --threads</b>   | (Optional; default to 1) Number of threads.                                   |
|                             | <b>-v, --verbose</b>   | (Optional) Verbose output.  |
|                             | <b>-n, --cnum</b>  | (Optional; default to 5000) Top level cell number.                            |
|                             | <b>-R, --ratio</b>   | (Optional; default to 20) Other level cell number ratio.                      |
|                             | <b>-a, --allocat</b>   | (Optional; default to 2) Allocation strategy.                                 |
|                             | <b>-g, --raw-gem</b>   | (Optional) Raw GEM file.  |
|                             | <b>-c, --canvas</b>  | (Optional; default to 0,0,90000,90000) Set canvas size, minX,minY,-maxX maxY. |
|                             | <b>-l, --limit</b>   | (Optional; default to 16,16) Set block limit.                                 |
|                             | <b>-S, --split</b>   | (Optional; default to 0) Split cellID to layers and blocks.                   |
| <b>-w, --errorCode-file</b> | (Optional; default to false) Set to turn on error code mode. |   |



Please check [2.14.1 Other applications of cellCut](#) to learn more about **cellCut**.

### 3.7.2 Usage Example

Run `cellCut` only if `register` aligned microscopic staining image is provided:

```
...
$ nucleusLayer=$(find /path/to/output/05.register -maxdepth 1 -name *fov_stitched_
transformed.tif -exec sh -c 'for f do basename -- "$f" _fov_stitched_transformed.
tif;done' sh {} + | grep -v IF)
$ nucleusMask=$(find /path/to/output/05.register -maxdepth 1 -name ${nucleusLayer}*
mask.tif)
$ mkdir -p /path/to/output/061P.cellcut
$ singularity exec SAW_v7.1.sif cellCut cgef \
  -i /path/to/output/04.calibration/{SN}.protein.calibrated.raw.gef \
  -m ${nucleusMask} \
  -o /path/to/output/061P.cellcut/{SN}.protein.cellbin.gef

$ mkdir -p /path/to/output/061T.cellcut
$ singularity exec SAW_v7.1.sif cellCut cgef \
  -i /path/to/output/04.calibration/{SN}.calibrated.raw.gef \
  -m ${nucleusMask} \
  -o /path/to/output/061T.cellcut/{SN}.cellbin.gef
```

### 3.7.3 Outputs

`cellCut` output files:

```
...
$ tree /path/to/output/061P.cellcut
/path/to/output/061P.cellcut
├── SN.protein.cellbin.gef

$ tree /path/to/output/061T.cellcut
/path/to/output/061T.cellcut
├── SN.cellbin.gef
```

## 3.8 cellCorrect

SAW **cellCorrect** pipeline runs to make adjustments based on the aligned cell segmentation image generated from **register** and **imageTools** and extract expression matrix of the adjusted one. **cellCorrect** outputs expression data in cell bin GEF and GEM formats. Run **cellCorrect** if results of cell correction are desired.

Running **cellCorrect** requires the following files:

- **calibration** output expression matrix file (**.calibrated.raw.gef**)
  - **register** and **imageTools** output cell segmentation mask TIFF file (.tif)
- 🕒 Expected running time for ~1G reads: ~2 min, Memory: ~10 G

### 3.8.1 Arguments and Options

Table 3-6 **cellCluster** Arguments and Options

| Parameter | Function  |
|-----------|---|
| <b>-i</b> | (Required) Input GEF file.  |
| <b>-m</b> | (Required) Input cell segmentation mask file.   |
| <b>-d</b> | (Required; default to 10) Outspread distance based on the cellular contour of cell segmentation image, in pixels. |
| <b>-o</b> | (Required) Path to output cell bin GEF, GEM and adjusted mask files.  |

### 3.8.2 Usage Example

Run **cellCorrect** as below:

```

$ nucleusLayer=$(find /path/to/output/05.register -maxdepth 1 -name *fov_stitched_
* transformed.tif -exec sh -c 'for f do basename -- "$f" _fov_stitched_transformed.
tif;done' sh {} + | grep -v IF)
$ nucleusMask=$(find /path/to/output/05.register -maxdepth 1 -name ${nucleusLayer}*
* mask.tif)

$ singularity exec SAW_v7.1.sif cellCorrect \
  -i /path/to/output/04.calibration/{SN}.protein.calibrated.raw.gef \
  -m ${nucleusMask} \
  -d 10 \
  -o /path/to/output/061P.cellcut

$ mv /path/to/output/061P.cellcut/{SN}.adjusted.cellbin.gef
* /path/to/output/061P.cellcut/{SN}.protein.adjusted.cellbin.gef
$ mv /path/to/output/061P.cellcut/{SN}.adjusted.gem /path/to/output/061P.cellcut/
* {SN}.protein.adjusted.gem

$ singularity exec SAW_v7.1.sif cellCorrect \
  -i /path/to/output/04.calibration/{SN}.calibrated.raw.gef \
  -m ${nucleusMask} \
  -d 10 \
  -o /path/to/output/061T.cellcut

```

### 3.8.3 Outputs

cellCorrect output files:

```
$ tree /path/to/output/061P.cellcut
/path/to/output/061P.cellcut
├── SN.protein.adjusted.cellbin.gef
├── <stainType>_SN_mask_edm_dis_10.tif
└── SN.protein.adjusted.gem
$ tree /path/to/output/061T.cellcut
/path/to/output/061T.cellcut
├── SN.adjusted.cellbin.gef
├── <stainType>_SN_mask_edm_dis_10.tif
└── SN.adjusted.gem
```

### 3.9 cellCluster-SP & cellCluster

SAW `cellCluster-SP` / `cellCluster` pipeline runs by clustering cells using the Leiden algorithm similarly to the procedures of `spatialCluster-SP` / `spatialCluster`. Run these two pipelines if cell bin results are desired.

Running `cellCluster-SP` / `cellCluster` requires the following files

- `cellCut` output cell bin expression matrix file (**.cellbin.gef**)
- 🕒 Expected running time for ~1G reads: ~5 min, Memory: ~5 G

#### 3.9.1 Arguments and Options

Table 3-7 `cellCluster-SP` and `cellCluster` Arguments and Options

| Parameter       | Function   |
|-----------------|--|
| <code>-i</code> | (Required) <code>cellCut</code> output cell bin gene expression matrix file. |
| <code>-o</code> | (Required) Output path to the clustering result in H5AD format.              |

#### 3.9.2 Usage Example

```

$ mkdir -p /path/to/output/071P.cellcluster
$ singularity exec SAW_v7.1.sif cellCluster-SP \
  -i /path/to/output/061P.cellcut/{SN}.protein.adjusted.cellbin.gef \
  -o /path/to/output/071P.cellcluster/{SN}.protein.adjusted.cell.cluster.h5ad
$ singularity exec SAW_v7.1.sif cellCluster-SP \
  -i /path/to/output/061P.cellcut/{SN}.protein.cellbin.gef \
  -o /path/to/output/071P.cellcluster/{SN}.protein.cell.cluster.h5ad

$ mkdir -p /path/to/output/071T.cellcluster
$ singularity exec SAW_v7.1.sif cellCluster \
  -i /path/to/output/061T.cellcut/{SN}.adjusted.cellbin.gef \
  -o /path/to/output/071T.cellcluster/{SN}.adjusted.cell.cluster.h5ad
$ singularity exec SAW_v7.1.sif cellCluster \
  -i /path/to/output/061T.cellcut/{SN}.cellbin.gef \
  -o /path/to/output/071T.cellcluster/{SN}.cell.cluster.h5ad

```

#### 3.9.3 Outputs

`cellCluster-SP` / `cellCluster` output files:

```

$ tree /path/to/output/071P.cellcluster
/path/to/output/071P.cellcluster
├── SN.protein.adjusted.cell.cluster.h5ad
└── SN.protein.cell.cluster.h5ad

$ tree /path/to/output/071T.cellcluster
/path/to/output/071T.cellcluster
├── SN.adjusted.cell.cluster.h5ad
└── SN.cell.cluster.h5ad

```

## 3.10 saturation

SAW **saturation** pipeline is performed to compute the sequencing saturation for the tissue coverage area.

Running **saturation** for Proteomics requires the following files:

- **mapping-SP** output statistical report includes CID mapping (**SN\_map.stat**)
- **mapping-SP** output saturation sampling file (**SN\_cid\_pid\_mid\_reads.tsv**)
- **tissueCut** output GEF file for the tissue coverage area (**.tissue.gef**)

Running **saturation** for Transcriptomics requires the following files:

- **mapping** output statistical report of CID mapping (**.stat**)
- **count** output saturation sampling file (**.txt**)
- **count** output statistical report of annotation (**.stat**)
- **tissueCut** output GEF file for the tissue coverage area (**.tissue.gef**)

🕒 Expected running time for ~1G reads: ~5 min, Memory: ~5 G

### 3.10.1 Arguments and Options

Table 3-8 **saturation** Arguments and Options

| Parameter        | Function   |
|------------------|--|
| <b>-i</b>        | (Required) <b>count</b> output saturation sampling file.   |
| <b>--tissue</b>  | (Required) <b>tissueCut</b> output GEF file for the tissue coverage area.  |
| <b>-o</b>        | (Required) Path to the directory where to store the <b>saturation</b> outputs. There has to be an existing path. |
| <b>--bcstat</b>  | (Required) <b>mapping</b> output statistical report of CID mapping. Separate multiple files by comma.            |
| <b>--summary</b> | (Required) <b>count</b> output statistical report of annotation.   |
| <b>--protein</b> | (Optional) Proteomics mode.  |



### 3.10.2 Usage Example

```

## Also adapt to muliple pairs of FASTQ files in Proteomics mode
$ mkdir -p /path/to/output/07P.saturation
$ singularity exec SAW_v7.1.sif saturation \
  -i /path/to/output/01P.mapping/{SN}_cid_pid_mid_reads.tsv \
  --tissue /path/to/output/06P.tissuecut/{SN}.protein.tissue.gef \
  -o /path/to/output/07P.saturation \
  --bcstat /path/to/output/01P.mapping/{lane}.CIDMap.stat \
  --protein

$ mkdir -p /path/to/output/07T.saturation
$ singularity exec SAW_v7.1.sif saturation \
  -i /path/to/output/03T.count/{SN}_raw_barcode_gene_exp.txt \
  --tissue /path/to/output/06T.tissuecut/{SN}.tissue.gef \
  -o /path/to/output/07T.saturation \
  --bcstat /path/to/output/01T.mapping/{lane}.CIDMap.stat \
  --summary /path/to/output/03T.count/
* {SN}.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam.summary.stat

```

\* Please be noted that these two lines belong to the same line of command.

For more than one pair of FASTQ files (Here showing an example of 2 pairs of FASTQ) in Transcriptomics:

```

$ mkdir -p /path/to/multi_lane_output/07T.saturation
$ singularity exec SAW_v7.1.sif saturation \
  -i /path/to/multi_lane_output/03T.count/{SN}_raw_barcode_gene_exp.txt \
  --tissue /path/to/multi_lane_output/06T.tissuecut/{SN}.tissue.gef \
  -o /path/to/multi_lane_output/07T.saturation \
  --bcstat /path/to/multi_lane_output/01T.mapping/{lane1}.CIDMap.stat,/path/to/
* multi_lane_output/01T.mapping/{lane2}.CIDMap.stat \
  --summary /path/to/multi_lane_output/03T.count/{SN}.Aligned.sortedByCoord.out.
* merge.q10.dedup.target.bam.summary.stat

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

### 3.10.3 Outputs

saturation output files:

```

$ tree /path/to/output/07P.saturation
├── plot_1x1_saturation.png
├── plot_200x200_saturation.png
└── sequence_saturation.tsv

$ tree /path/to/output/07T.saturation
├── plot_1x1_saturation.png
├── plot_200x200_saturation.png
└── sequence_saturation.tsv

```

## 3.11 multiomicsAnalysis

SAW `multiomicsAnalysis` pipeline integrates gene and protein data and computes the latent space by Total Variational Inference<sup>14</sup>. Perform clustering analysis for latent space and do one-vs-all differential expression analysis to find marker genes and output proteins expression heat map within leiden cluster.

Running `multiomicsAnalysis` requires the following files:

- `tissueCut` output tissue-covered expression matrix (**.tissue.gef / .tissue.gem.gz**)
- or `cellCut` output cell bin expression matrix (**.cellbin.gef**)
- Protein database file
- 🕒 Expected running time for ~1G reads: ~1 h, Memory: ~120 G



Please confirm that the input protein reference file only records the input proteins. You can get protein database in SAW Github.

### 3.11.1 Arguments and Options

Table 3-9 `multiomicsAnalysis` Arguments and Options

| Parameter              | Function   |
|------------------------|--|
| <code>-r</code>        | (Required) Path to Transcriptomics GEM/GEF/Cellbin GEF file.   |
| <code>-p</code>        | (Required) Path to Proteomics GEM/GEF/Cellbin GEF file.  |
| <code>-b</code>        | (Required) Bin size. '0' represents cell bin mode.   |
| <code>-pl</code>       | (Required) Path to the protein database file.  |
| <code>-o</code>        | (Required) The output directory.   |
| <code>--use_gpu</code> | (Optional, <b>default = auto</b> ) Prioritize GPU use if set <b>auto</b> . <b>true/false</b> represents use/ do not use GPU.   |
| <code>--gpu</code>     | (Optional, <b>default = auto</b> ) Set the GPU index, e.g, <code>--gpu=0</code> . <b>auto</b> represents the system allocates the index. Valid for <code>--use_gpu=true</code> . |

### 3.11.2 Usage Example

Run `multiomicsAnalysis` in square bin mode.

Input GEM case:

```

$ mkdir -p /path/to/output/08.multiomics
## Convert tissue.gef to tissue.gem by cellCut view. You can find usage in
2.15.1 Other applications of cellCut

$ singularity exec SAW_v7.1.sif multiomicsAnalysis \
  -r /path/to/output/06T.tissuecut/{SN}.tissue.gem.gz \
  -p /path/to/output/06P.tissuecut/{SN}.protein.tissue.gem.gz \
  -b 50 \
  -pl /path/to/data/{SN}.protein.database.txt \ ## you can use protein
  panel in SAW Github directly
  -o /path/to/output/08.multiomics

```

Input GEF case:

```
$ mkdir -p /path/to/output/08.multiomics
$ singularity exec SAW_v7.1.sif multiomicsAnalysis \
  -r /path/to/output/06T.tissuecut/{SN}.tissue.gef \
  -p /path/to/output/06P.tissuecut/{SN}.protein.tissue.gef \
  -b 50 \
  -pl /path/to/data/{SN}.protein.database.txt \
  -o /path/to/output/08.multiomics
```

Run `multiomicsAnalysis` in cell bin mode:

```
$ mkdir -p /path/to/output/08.multiomics
$ singularity exec SAW_v7.1.sif multiomicsAnalysis \
  -r /path/to/output/061T.cellcut/{SN}.adjusted.cellbin.gef \
  -p /path/to/output/061P.cellcut/{SN}.protein.adjusted.cellbin.gef \
  -b 50 \
  -pl /path/to/data/{SN}.protein.database.txt \
  -o /path/to/output/08.multiomics
```

### 3.11.3 Outputs

`multiomicsAnalysis` square bin mode output files are:

```
$ tree /path/to/output/08.multiomics
/path/to/output/08.multiomics
├── SN_50_differential_expression.csv
├── SN_50_dotplot_RNA_totalVI_03.png
├── SN_50.h5mu
├── SN_50_matrixplot_Protein_totalVI_04.png
├── SN_50_Protein_Correlation_Heatmap_05.png
├── SN_50_spatial_leiden_totalVI_02.png
└── SN_50_UMAP_leiden_totalVI_01.png
```

`multiomicsAnalysis` cell bin mode output files are:

```
$ tree /path/to/output/08.multiomics
/path/to/output/08.multiomics
├── SN_0_differential_expression.csv
├── SN_0_dotplot_RNA_totalVI_03.png
├── SN_0.h5mu
├── SN_0_matrixplot_Protein_totalVI_04.png
├── SN_0_Protein_Correlation_Heatmap_05.png
├── SN_0_spatial_leiden_totalVI_02.png
└── SN_0_UMAP_leiden_totalVI_01.png
```

## 3.12 report-PT

SAW **report-PT** pipeline is performed to integrate analysis results from each step and generate the report in JSON format as well as a web report in HTML format. HTML analytical report integrate genes and proteins' spatial expression distribution, key statistical metrics, sequencing saturation plots, clustering analysis results, image processing information and multiomics analysis.

Running **report-PT** requires the following files and information:

- **mapping** output statistical report of CID mapping and **STAR** alignment (**.stat, .Log.final.out**)
- **mapping-SP** output statistical report of CID mapping and PID alignment (**.stat**)
- **count** output statistical report of annotation (**.stat**)
- **register** processed image process record file and image pyramid RPI file (**.ipr, .rpi**)
- **tissueCut** and **cellCut** (if available) output GEF file, statistical report of tissue-covered region, plots and image pyramid RPI file (**.gef, .stat, .png**)
- **spatialCluster** & **spatialCluster-SP** and **cellCluster** & **cellCluster-SP** (if available) output clustering H5AD file (**.h5ad**)
- **saturation** output Transcriptomics bin200 sequence saturation plot (**.png**)
- **multiomicsAnalysis** output Transcriptomics bin200 sequence saturation plot (**.png**)
- species, tissue, and reference information

🕒 Expected running time for ~1G reads: ~1 min, Memory: 1G

### 3.12.1 Arguments and Options

Table 3-10 **report-PT** Arguments and Options

| Parameter                         | Function  |
|-----------------------------------|---|
| <b>--RNAMapStat</b>               | (Required) <b>mapping</b> output statistical report of CID mapping (.CIDMap.stat). Separate multiple files by comma.  |
| <b>--ProteinMapStat</b>           | (Required) <b>mapping-SP</b> output statistical report of CID mapping and PID alignment (*_map.stat).                 |
| <b>--a</b>                        | (Required) <b>mapping</b> output statistical report of STAR alignment (.final.out). Separate multiple files by comma. |
| <b>-g</b>                         | (Required) <b>count</b> output statistical report of annotation (.summary.stat).                                      |
| <b>--RNATissueCutStat</b>         | (Required) <b>tissueCut</b> output Transcriptomics statistical report for tissue-covered regions.                     |
| <b>--ProteinTissueCutStat</b>     | (Required) <b>tissueCut</b> output Proteomics statistical report for tissue-covered regions.                          |
| <b>--RNAVisGef</b>                | (Required) Visible square bin GEF file for Transcriptomics (SN.gef), which contains group /wholeExp/.                 |
| <b>--ProteinVisGef</b>            | (Required) Visible square bin GEF file for Proteomics (SN.protein.gef), which contains group /wholeExp/.              |
| <b>--RNASquareClusterFile</b>     | (Required) <b>spatialCluster</b> output H5AD file.  |
| <b>--ProteinSquareClusterFile</b> | (Required) <b>spatialCluster-SP</b> output H5AD file.   |

| Parameter                          | Function  |
|------------------------------------|---|
| <code>--rpi</code>                 | (Optional) The image pyramid RPI file.  |
| <code>--saturation</code>          | (Required) <b>saturation</b> output Transcriptomics bin 200 sequence saturation plot.   |
| <code>--sn</code>                  | (Required) The Stereo-seq Chip T serial number.   |
| <code>--RNACellGef</code>          | (Optional) The cell bin GEF file for Transcriptomics.   |
| <code>--ProteinCellGef</code>      | (Optional) The cell bin GEF file for Proteomics.  |
| <code>--RNACellCluster</code>      | (Optional) <b>cellCluster</b> output H5AD file.   |
| <code>--ProteinCellCluster</code>  | (Optional) <b>cellCluster-SP</b> output H5AD file.  |
| <code>--iprFile</code>             | (Optional) A processed IPR (image process record) file.   |
| <code>--species</code>             | (Required) A string of species name.  |
| <code>--tissue</code>              | (Required) A string of tissue type.   |
| <code>--rna_tissue_fig</code>      | (Required) <b>tissueCut</b> statistics plots directory for Transcriptomics.   |
| <code>--protein_tissue_fig</code>  | (Required) <b>tissueCut</b> statistics plots directory for Proteomics.  |
| <code>--reference</code>           | (Required) A string of reference used for <b>mapping</b> .  |
| <code>--pipelineVersion</code>     | (Required) Set to specifying analysis pipeline version.   |
| <code>--adt_fastq_name</code>      | (Required) Stomics-ADT fastq file name. Separated by comma.   |
| <code>--multimomics_spatial</code> | (Optional) <b>multimomicsAnalysis</b> output spatial cluster plot.  |
| <code>--multimomics_umap</code>    | (Optional) <b>multimomicsAnalysis</b> output UMAP plot.   |
| <code>--multimomics_heatmap</code> | (Optional) <b>multimomicsAnalysis</b> output expression heatmap of proteins per leiden cluster.   |
| <code>--multimomics_bubble</code>  | (Optional) <b>multimomicsAnalysis</b> output expression heatmap and fraction of cells (spots) of genes per leiden cluster.  |
| <code>--qc_metrics</code>          | (Optional) The customized QC-metrics file (.xlsx) for SAW. QC-metrics focus on sequencing quality, and performance of in situ restoration of sequencing reads. For example, "Total Q30" and "Fraction MID in Spots Under Tissue". |
| <code>--o</code>                   | (Required) The directory to store outputs.  |
| <code>--inputYaml</code>           | (Optional) <b>report-PT</b> output input.yaml   |

### 3.12.2 Usage Example



**Please Note! Replace `{SN}`, `{lane}`, `{species_name}`, `{tissue_type}`, `{reference_index}` with the real information.**

Completion of GEFs:

```

$ imageIPR=$(find /path/to/output/05.register -maxdepth 1 -name {SN}*.ipr | head -1)
## has to be a processed IPR
$ singularity exec SAW_v7.1.sif cellCut bgef \
  -i /path/to/output/04.calibration/{SN}.calibrated.raw.gef \
  -o /path/to/output/04.calibration/{SN}.gef \
  -b 1,10,20,50,100,200,500 \
  -O Transcriptomics

$ singularity exec SAW_v7.1.sif cellCut bgef \
  -i /path/to/output/04.calibration/{SN}.protein.calibrated.raw.gef \
  -o /path/to/output/04.calibration/{SN}.protein.gef \
  -b 1,5,10,20,50,100,150,200 \
  -O Proteomics

```

Run `report-PT` if `register` aligned microscopic staining image is provided and has cell bin output:

```

...
$ mkdir -p /path/to/output/10.report
$ singularity exec SAW_v7.1.sif report-PT \
  --RNAMapStat /path/to/output/01T.mapping/{lane}.CIDMap.stat \
  --ProteinMapStat /path/to/output/01P.mapping/{SN}_map.stat \
  -a /path/to/output/01T.mapping/{lane}.Log.final.out \
  -g /path/to/output/03T.count/
* {SN}.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam.summary.stat \
  --RNATissueCutStat /path/to/output/06T.tissuecut/tissuecut.stat \
  --ProteinTissueCutStat /path/to/output/06P.tissuecut/tissuecut.stat \
  --RNAVisGef /path/to/output/04.calibration/{SN}.gef \
  --ProteinVisGef /path/to/output/04.calibration/{SN}.protein.gef \
  --RNASquareClusterFile /path/to/output/07T.spatialcluster/
* {SN}.bin200_1.0.spatial.cluster.h5ad \
  --ProteinSquareClusterFile /path/to/output/07P.spatialcluster/
{SN}.protein.bin200_0.1.spatial.cluster.h5ad \
  --rpi /path/to/output/05.register/{SN}.rpi \
  --saturation /path/to/output/08.saturation/plot_200x200_saturation.png \
  --sn {SN} \
  --RNACellGef /path/to/output/061T.cellcut/{SN}.adjusted.cellbin.gef \
  --ProteinCellGef /path/to/output/061P.cellcut/
* {SN}.protein.adjusted.cellbin.gef \
  --RNACellCluster /path/to/output/071T.cellcluster/
* {SN}.adjusted.cell.cluster.h5ad \
  --ProteinCellCluster /path/to/output/071P.cellcluster/
{SN}.protein.adjusted.cell.cluster.h5ad \
  --iprFile ${imageIPR} \
  --species {species_name} \
  --tissue {tissue_type} \
  --rna_tissue_fig /path/to/output/06T.tissuecut/tissue_fig \
  --protein_tissue_fig /path/to/output/06P.tissuecut/tissue_fig \
  --reference {reference_index} \
  --pipelineVersion SAW_v7.1.0 \
  --adt_fastq_name /path/to/data/{lane}_read_1.fq.gz \
  --multimomics_spatial /path/to/output/09.mutiomics/
* {SN}_50_spatial_leiden_totalVI_02.png \
  --multimomics_umap /path/to/output/09.mutiomics/
* {SN}_50_UMAP_leiden_totalVI_01.png \
  --multimomics_heatmap /path/to/output/09.mutiomics/
{SN}_50_matrixplot_Protein_totalVI_04.png \
  --multimomics_bubble /path/to/output/09.mutiomics/
* {SN}_50_dotplot_RNA_totalVI_03.png \
  -o /path/to/output/10.report

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.



For more than one pair of FASTQ files (Here showing an example of 2 pairs of FASTQ in STOmics-RNA and STOmics-ADT respectively),

```

$ mkdir -p /path/to/multi_lane_output/10.report
$ singularity exec SAW_v7.1.sif report-PT \
  --RNAMapStat /path/to/multi_lane_output/01T.mapping/{lane1}.CIDMap.stat,
/path/to/multi_lane_output/01T.mapping/{lane2}.CIDMap.stat \
  --ProteinMapStat /path/to/multi_lane_output/01P.mapping/{SN}_map.stat \
  -a /path/to/multi_lane_output/01T.mapping/{lane1}.Log.final.out,
/path/to/multi_lane_output/01T.mapping/{lane2}.Log.final.out \
  -g /path/to/multi_lane_output/03T.count/
{SN}.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam.summary.stat \
  --RNATissueCutStat /path/to/multi_lane_output/06T.tissuecut/tissuecut.stat \
  --ProteinTissueCutStat
/path/to/multi_lane_output/06P.tissuecut/tissuecut.stat \
  --RNAVisGef /path/to/multi_lane_output/04.calibration/{SN}.gef \
  --ProteinVisGef /path/to/multi_lane_output/04.calibration/{SN}.protein.gef \
  --RNASquareClusterFile /path/to/multi_lane_output/07T.spatialcluster/
{SN}.bin200_1.0.spatial.cluster.h5ad \
  --ProteinSquareClusterFile /path/to/multi_lane_output/07P.spatialcluster/
{SN}.protein.bin200_0.1.spatial.cluster.h5ad \
  --rpi /path/to/multi_lane_output/05.register/{SN}.rpi \
  --saturation
/path/to/multi_lane_output/08.saturation/plot_200x200_saturation.png \
  --sn {SN} \
  --RNACellGef /path/to/multi_lane_output/061T.cellcut/
{SN}.adjusted.cellbin.gef \
  --ProteinCellGef /path/to/multi_lane_output/061P.cellcut/
{SN}.protein.adjusted.cellbin.gef \
  --RNACellCluster /path/to/multi_lane_output/071T.cellcluster/
{SN}.adjusted.cell.cluster.h5ad \
  --ProteinCellCluster /path/to/multi_lane_output/071P.cellcluster/
{SN}.protein.adjusted.cell.cluster.h5ad \
  --iprFile ${imageIPR} \
  --species {species_name} \
  --tissue {tissue_type} \
  --rna_tissue_fig /path/to/multi_lane_output/06T.tissuecut/tissue_fig \
  --protein_tissue_fig /path/to/multi_lane_output/06P.tissuecut/tissue_fig \
  --reference {reference_index} \
  --pipelineVersion SAW_v7.1.0 \
  --adt_fastq_name /path/to/data/{lane1}_read_1.fq.gz,/path/to/data/
{lane2}_read_1.fq.gz \
  --multimomics_spatial /path/to/multi_lane_output/09.multimomics/
{SN}_50_spatial_leiden_totalVI_02.png \
  --multimomics_umap /path/to/multi_lane_output/09.multimomics/
{SN}_50_UMAP_leiden_totalVI_01.png \

```



```

...
--multimomics_heatmap /path/to/multi_lane_output/09.multimomics/
{SN}_50_matrixplot_Protein_totalVI_04.png \
--multimomics_bubble /path/to/multi_lane_output/09.multimomics/
{SN}_50_dotplot_RNA_totalVI_03.png \
-o /path/to/multi_lane_output/10.report

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

Run report-PT for register outputs in which the microscopic image has registered with gene expression matrix but did not perform cell segmentation. Here is an example of just one pair of FASTQ,

```

...
$ mkdir -p /path/to/output/10.report
$ singularity exec SAW_v7.1.sif report-PT \
--RNAMapStat /path/to/output/01T.mapping/{lane}.CIDMap.stat \
--ProteinMapStat /path/to/output/01P.mapping/{SN}_map.stat \
-a /path/to/output/01T.mapping/{lane}.Log.final.out \
-g /path/to/output/03T.count/
{SN}.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam.summary.stat \
--RNATissueCutStat /path/to/output/06T.tissuecut/tissuecut.stat \
--ProteinTissueCutStat /path/to/output/06P.tissuecut/tissuecut.stat \
--RNAVisGef /path/to/output/04.calibration/{SN}.gef \
--ProteinVisGef /path/to/output/04.calibration/{SN}.protein.gef \
--RNASquareClusterFile /path/to/output/07T.spatialcluster/
{SN}.bin200_1.0.spatial.cluster.h5ad \
--ProteinSquareClusterFile /path/to/output/07P.spatialcluster/
{SN}.protein.bin200_0.1.spatial.cluster.h5ad \
--rpi /path/to/output/05.register/{SN}.rpi \
--saturation /path/to/output/08.saturation/plot_200x200_saturation.png \
--sn {SN} \
--iprFile ${imageIPR} \
--species {species_name} \
--tissue {tissue_type} \
--rna_tissue_fig /path/to/output/06T.tissuecut/tissue_fig \
--protein_tissue_fig /path/to/output/06P.tissuecut/tissue_fig \
--reference {reference_index} \
--pipelineVersion SAW_v7.1.0 \
--adt_fastq_name /path/to/data/{lane}_read_1.fq.gz \
--multimomics_spatial /path/to/output/09.multimomics/
{SN}_50_spatial_leiden_totalVI_02.png \
--multimomics_umap /path/to/output/09.multimomics/
{SN}_50_UMAP_leiden_totalVI_01.png \
--multimomics_heatmap /path/to/output/09.multimomics/
{SN}_50_matrixplot_Protein_totalVI_04.png \
--multimomics_bubble /path/to/output/09.multimomics/
{SN}_50_dotplot_RNA_totalVI_03.png \
-o /path/to/output/10.report

```





Run `report-PT` if `register` aligned microscopic staining image is absent (Here shows an example of just one pair of FASTQ, similar to multiple pairs),

```

...
$ mkdir -p /path/to/output/10.report
$ singularity exec SAW_v7.1.sif report-PT \
  --RNAMapStat /path/to/output/01T.mapping/{lane}.CIDMap.stat \
  --ProteinMapStat /path/to/output/01P.mapping/{SN}_map.stat \
  -a /path/to/output/01T.mapping/{lane}.Log.final.out \
  -g /path/to/output/03T.count/
* {SN}.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam.summary.stat \
  --RNATissueCutStat /path/to/output/06T.tissuecut/tissuecut.stat \
  --ProteinTissueCutStat /path/to/output/06P.tissuecut/tissuecut.stat \
  --RNAVisGef /path/to/output/04.calibration/{SN}.gef \
  --ProteinVisGef /path/to/output/04.calibration/{SN}.protein.gef \
  --RNASquareClusterFile /path/to/output/07T.spatialcluster/
* {SN}.bin200_1.0.spatial.cluster.h5ad \
  --ProteinSquareClusterFile /path/to/output/07P.spatialcluster/
* {SN}.protein.bin200_0.1.spatial.cluster.h5ad \
  --saturation /path/to/output/08.saturation/plot_200x200_saturation.png \
  --sn {SN} \
  --species {species_name} \
  --tissue {tissue_type} \
  --rna_tissue_fig /path/to/output/06T.tissuecut/tissue_fig \
  --protein_tissue_fig /path/to/output/06P.tissuecut/tissue_fig \
  --reference {reference_index} \
  --pipelineVersion SAW_v7.1.0 \
  --adt_fastq_name /path/to/data/{lane}_read_1.fq.gz \
  --multimomics_spatial /path/to/output/09.multimomics/
* {SN}_50_spatial_leiden_totalVI_02.png \
  --multimomics_umap /path/to/output/09.multimomics/
* {SN}_50_UMAP_leiden_totalVI_01.png \
  --multimomics_heatmap /path/to/output/09.multimomics/
* {SN}_50_matrixplot_Protein_totalVI_04.png \
  --multimomics_bubble /path/to/output/09.multimomics/
* {SN}_50_dotplot_RNA_totalVI_03.png \
  -o /path/to/output/10.report

```

\* Please be noted that we use the backward slash “\” to indicate the end of a line in a command that spans multiple lines.

## 2.14.3 Outputs

**report-PT** output files that have cell bin data input are organized as below:

```
$ tree /path/to/output/10.report
/path/to/output/10.report
├── AnalysisReport
├── SN.statistics.json
├── input.yaml
├── protein_cell
└── rna_cell
```

**report-PT** output files that the cell bin data inputs are absent:

```
$ tree /path/to/output/10.report
/path/to/output/10.report
├── AnalysisReport
├── SN.statistics.json
└── input.yaml
```

# CHAPTER 4

TEST DATA

DEMONSTRATION

—

TRANSCRIPTOMICS

Users may refer to this section as a format for testing SAW process, of the files shown in this chapter as the reference in testing SAW pipelines. This chapter includes the statistics results and examples of critical files for each key step.

SN: SS200000135TL\_D1



“...” in the demo stands for some lines of log information that can be omitted.

## 4.1 mapping

### 4.1.1 Statistical Report for CID Mapping and Filtering

```
$ cat /path/to/output/01.mapping/E100026571_L01_trim_read_1.CIDMap.stat
...
        unique_CID_in_mask:           645784920
        unique_CID_in_fq:             78021796
        total_reads:                   1002214171
        CID_with_N_reads:              8031                0.00 %
        CID_mapped_reads:              845113606           84.32 %
        CID_exactly_mapped_reads:      698277934           69.67 %
        CID_mapped_reads_with_mismatch: 146827641           14.65 %
        discarded_MID_reads:           8451158             0.84 %
        MID_with_N_reads:              13339               0.00 %
        MID_with_polyA_reads:          13044               0.00 %
        MID_with_low_quality_base_reads: 8424775             0.84 %
        reads_with_dnb:                45281336            4.52 %
        reads_with_adapter:            8136836             0.81 %
        short_reads_filtered_with_polyA: 14191622            1.42 %
        reads_with_polyA:              105849530           10.56 %
        reads_with_rRNA:               0                   0.00 %
        Q10_bases_in_seq:              99.47 %
        Q20_bases_in_seq:              97.12 %
        Q30_bases_in_seq:              91.08 %
        Q10_bases_in_MID:              99.26 %
        Q20_bases_in_MID:              96.32 %
        Q30_bases_in_MID:              89.45 %
        Q10_bases_in_CID:              99.54 %
        Q20_bases_in_CID:              97.49 %
        Q30_bases_in_CID:              91.74 %
```

## 4.1.2 Statistical Report for Reference Genome Alignment

```

$ cat /path/to/output/01.mapping/E100026571_L01_trim_read_1.Log.final.out
...
      Number of input reads |           769052654
      Average input read length |           95
      UNIQUE READS:
      Uniquely mapped reads number |        645319822
      Uniquely mapped reads % |        83.91%
      Average mapped length |        95.14
      Number of splices: Total |        67634124
      Number of splices: Annotated (sjdb) |        65711163
      Number of splices: GT/AG |        66445242
      Number of splices: GC/AG |        457783
      Number of splices: AT/AC |        41573
      Number of splices: Non-canonical |        689526
      Mismatch rate per base, % |        0.50%
      Deletion rate per base |        0.07%
      Deletion average length |        3.91
      Insertion rate per base |        0.03%
      Insertion average length |        1.25
      MULTI-MAPPING READS:
      Number of reads mapped to multiple loci |        87828411
      % of reads mapped to multiple loci |        11.42%
      Number of reads mapped to too many loci |        5333051
      % of reads mapped to too many loci |        0.69%
      UNMAPPED READS:
      Number of reads unmapped: too many mismatches |        0
      % of reads unmapped: too many mismatches |        0.00%
      Number of reads unmapped: too short |        29365488
      % of reads unmapped: too short |        3.82%
      Number of reads unmapped: other |        1205882
      % of reads unmapped: other |        0.16%
      CHIMERIC READS:
      Number of chimeric reads |        0
      % of chimeric reads |        0.00%

```

## 4.1.3 Example of BAM mapping

```

$ samtools view /path/to/output/01.mapping/E100026571_L01_trim_read_1.Aligned.
sortedByCoord.out.bam | head -2
E100026571L1C007R00303973559    256    1    3000644 3    100M    *    0
0    GCCTCATTGTGCCCATATGTTTGCCTATGTTGTGGACTTATTTTCATTAACCTTTAAAACATCTTTA-
ATTTTTTCTTTATTTTCATCATTGACCAAGCT    -FCA9D?GFFD<-D<CGFEGD-DG*FGFDFBE;E(9BGGE38FFF-
G9GG;0?GGFGB?E@G:GGG3GF79F0GGDG?G<D>F;EG,G?<<CD4>G=>B+C    NH:i:2    HI:i:2    AS:i:94
nM:i:2    Cx:i:8839    Cy:i:7539    UR:Z:120CF
E100026571L1C003R03702347721    0    1    3001778 255    100M    *    0
0    GTATGACATCTGTCCAGGATCTTCTAGCTTTTCATAGTCTCTGGTGAGAAGTCTGGAGTAATTCTAATAGG-
CCTGCATTTATATGTTACTTGACCTTTTTTC    EEFEDFFEFFFEFFFEFFEC@EFFFFDFFEEFFFEFFFCFEFFAFB-
FCED??FGBEFFDC:FFFDCFAF4FAFFDFFDGD?DFBD.F@FECA/FEDEFFAA    NH:i:1    HI:i:1    AS:i:92
nM:i:3    Cx:i:12136    Cy:i:14034    UR:Z:C0808

```

## 4.2 merge

### 4.2.1 Example of Mapped CID List with Reads Count File

```

$ head /path/to/output/02.merge/SS200000135TL_D1.merge.barcodeReadsCount.txt
7127      18002     48
4348      19028     1
14130     8635      1
7618      14537     24
4912      10945     5
16783     12914     1
15539     8177      1
9288      8082      14
7274      16533     59
9087      10657     10

```

## 4.3 count

### 4.3.1 Statistical Report for MID Filtering and Gene Annotation

```

$ cat /path/to/output/03.count/SS200000135TL_D1.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam.summary.stat
## FILTER & DEDUPLICATION METRICS
TOTAL_READS      PASS_FILTER      ANNOTATED_READS  UNIQUE_READS      FAIL_FILTER_RATE
FAIL_ANNOTATE_RATE  DUPLICATION_RATE
733148233        645319822        533743961        108363128         11.98   17.29   79.70
## ANNOTATION METRICS
TOTAL_READS      MAP              EXONIC           INTRONIC          INTERGENIC        TRANSCRIP-
TOME  ANTISENSE
645319822        645319822        484480416        49263545          111575861         533743961
110185975
100.0            100.0            75.1              7.6                17.3              82.7
17.1

```

### 4.3.2 Example of Annotated BAM

```

$ samtools view /path/to/output/03.count/SS200000135TL_D1.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam | head -2
E100026571L1C007R00303973559      768      1      3000644 3      100M      *      0      0
GCCTCATTGTGCCCATATGTTTGCCTATGTTGTGGACTTATTTTCATTAACCTTTAAAACATCTTTAATTTTTTTCTTTATTTTCAT-
CATTGACCAAGCT      -FCA9D?GFFD<-D<CGFEGD-DG*FGFDFBE;E(9BGGE38FFFG9GG;0?GGFGB?E@G:GGG3GF79F-
0GGDG?G<D>F;EG,G?<<CD4>G=>B+C      NH:i:2  HI:i:1  AS:i:94  nM:i:2  Cx:i:8839      Cy:i:7539
UR:Z:120CF
E100026571L1C003R03702347721      0      1      3001778 255      100M      *      0      0
GTATGACATCTGTCCAGGATCTTCTAGCTTTCATAGTCTCTGGTGAGAAGTCTGGAGTAATTCTAATAGGCCTGCATTATAT-
GTTACTTGACCTTTTTC      EEFEDFFEFFFEFFFEFFEC@EFFFFDFFEFFFEFFFCFEFFAFBFCED??FGBEFFDC:FFF-
DCFAF4FAFFDFFDG?DFBD.F@FECA/FEDEFFAA      NH:i:1  HI:i:1  AS:i:92  nM:i:3  Cx:i:12136
Cy:i:14034      UR:Z:C0808      XF:i:2

```

### 4.3.3 Example of count Gene Expression Matrix

```

$ h5dump -n /path/to/output/03.count/SS200000135TL_D1.raw.gef
HDF5 "/path/to/output/03.count/SS200000135TL_D1.raw.gef" {
FILE_CONTENTS {
  group /
  group /geneExp
  group /geneExp/bin1
  dataset /geneExp/bin1/exon
  dataset /geneExp/bin1/expression
  dataset /geneExp/bin1/gene
}
}

$ h5dump -d /geneExp/bin1/expression /path/to/output/03.count/SS200000135TL_
D1.raw.gef | head -15
HDF5 "/path/to/output/03.count/SS200000135TL_D1.raw.gef" {
DATASET "/geneExp/bin1/expression" {
  DATATYPE H5T_COMPOUND {
    H5T_STD_U32LE "x";
    H5T_STD_U32LE "y";
    H5T_STD_U8LE "count";
  }
  DATASPACE SIMPLE { ( 76210618 ) / ( 76210618 ) }
  DATA {
    (0): {
      636,
      12671,
      2
    },
    (1): {

```

```

$ h5dump -d /geneExp/bin1/gene /path/to/output/03.count/SS200000135TL_D1.raw.gef |
head -20
HDF5 "/path/to/output/03.count/SS200000135TL_D1.raw.gef" {
DATASET "/geneExp/bin1/gene" {
  DATATYPE H5T_COMPOUND {
    H5T_STRING {
      STRSIZE 32;
      STRPAD H5T_STR_NULLTERM;
      CSET H5T_CSET_ASCII;
      CTYPE H5T_C_S1;
    } "gene";
    H5T_STD_U32LE "offset";
    H5T_STD_U32LE "count";
  }
  DATASPACE SIMPLE { ( 24670 ) / ( 24670 ) }
  DATA {
    (0): {
      "0610005C13Rik",
      0,
      45
    },
    (1): {

```

### 4.3.4 Example of count Sampling File

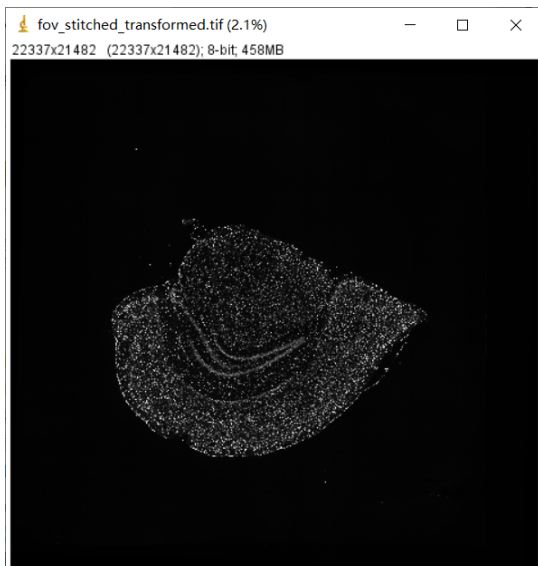
```
$ head -8 /path/to/output/03.count/SS200000135TL_D1_raw_barcode_gene_exp.txt
x y geneIndex MIDIndex readCount
9602 7705 10551 611723 2
4888 10392 10551 665954 4
8901 7096 10551 881671 1
8901 7096 10551 357383 20
7397 18783 10551 355789 1
9155 13032 10551 297666 1
9155 13032 10551 298690 1
```

## 4.4 register and imageTools

### 4.4.1 Registered image

File /path/to/output/04.register/ssDNA\_fov\_stitched\_transformed.tif and /path/to/output/04.register/ssDNA\_SS200000135TL\_D1\_regist.tif.

/path/to/output/04.register/ssDNA\_fov\_stitched\_transformed.tif



/path/to/output/04.register/ssDNA\_SS200000135TL\_D1\_regist.tif





## 4.4.2 Image Process Record File

```

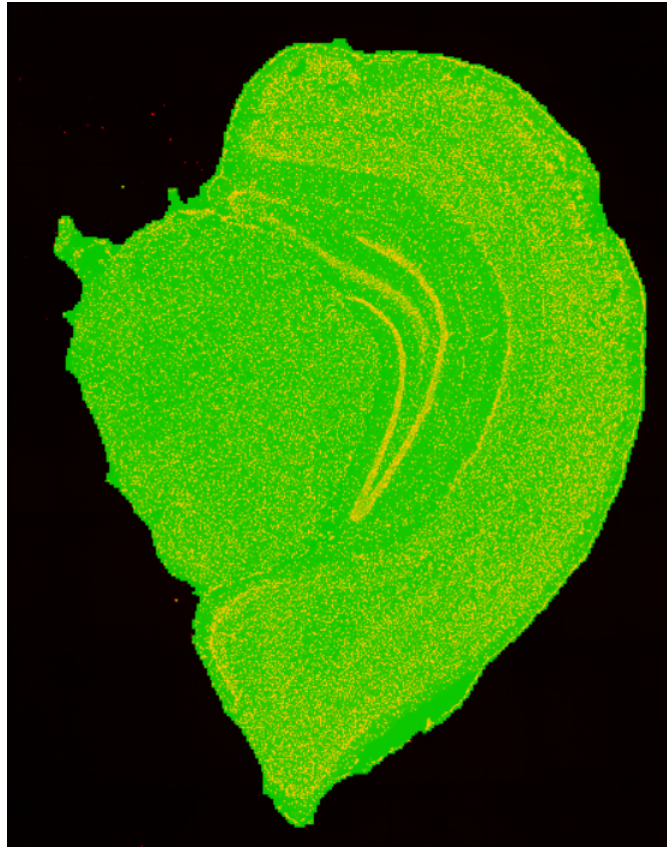
$ h5dump -n /path/to/output/04.register/SS200000135TL_D1_20220527_201353_1.1.0.ipr
HDF5 "/path/to/output/04.register/SS200000135TL_D1_20220527_201353_1.1.0.ipr" {
FILE_CONTENTS {
  group      /
  group      /ManualState
  dataset    /Preview
  group      /StereoResepSwitch
  group      /ssDNA
  group      /ssDNA/CellSeg
  dataset    /ssDNA/CellSeg/CellMask
  dataset    /ssDNA/CellSeg/CellSegTrace
  group      /ssDNA/ImageInfo
  dataset    /ssDNA/ImageInfo/RGBScale
  group      /ssDNA/QCInfo
  group      /ssDNA/QCInfo/CrossPoints
  dataset    /ssDNA/QCInfo/CrossPoints/0_0
  ...
  dataset    /ssDNA/QCInfo/CrossPoints/9_8
  group      /ssDNA/Register
  dataset    /ssDNA/Register/MatrixTemplate
  group      /ssDNA/Stitch
  group      /ssDNA/Stitch/ScopeStitch
  dataset    /ssDNA/Stitch/ScopeStitch/GlobalLoc
  dataset    /ssDNA/Stitch/ScopeStitch/ScopeHorizontalJitter
  dataset    /ssDNA/Stitch/ScopeStitch/ScopeJitterDiff
  dataset    /ssDNA/Stitch/ScopeStitch/ScopeVerticalJitter
  group      /ssDNA/Stitch/StitchEval
  dataset    /ssDNA/Stitch/StitchEval/GlobalDeviation
  dataset    /ssDNA/Stitch/StitchEval/StitchEvalH
  dataset    /ssDNA/Stitch/StitchEval/StitchEvalV
  dataset    /ssDNA/Stitch/TemplatePoint
  dataset    /ssDNA/Stitch/TransformTemplate
  group      /ssDNA/TissueSeg
  dataset    /ssDNA/TissueSeg/TissueMask
}
}

$ h5dump -A /path/to/output/04.register/SS200000135TL_D1_20220527_201353_1.1.0.ipr
| head -20
HDF5 "/path/to/output/04.register/SS200000135TL_D1_20220527_201353_1.1.0.ipr" {
GROUP "/" {
  ATTRIBUTE "IPRVersion" {
    DATATYPE H5T_STRING {
      STRSIZE H5T_VARIABLE;
      STRPAD H5T_STR_NULLTERM;
      CSET H5T_CSET_UTF8;
      CTYPE H5T_C_S1;
    }
    DATASPACE SCALAR
    DATA {
      (0): "0.2.0"
    }
  }
  GROUP "ManualState" {
    ATTRIBUTE "cellseg" {
      DATATYPE H5T_ENUM {
        H5T_STD_I8LE;
        "FALSE"      0;
        "TRUE"       1;
      }
    }
  }
}
}

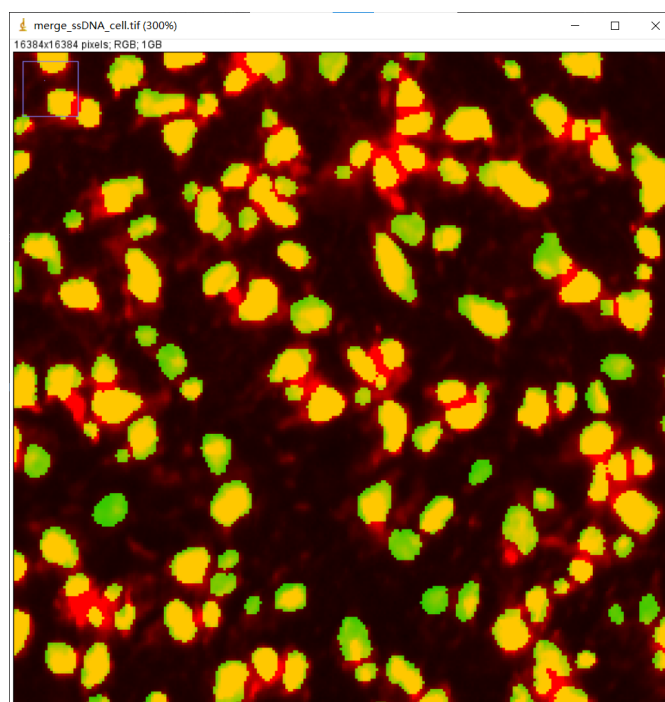
```

### 4.4.3 ImageTools merge

Merged image of microscopy image `ssDNA_SS200000135TL_D1_regist.tif` and tissue segmentation mask file `ssDNA_SS200000135TL_D1_tissue_cut.tif` to check tissue segmentation performance.

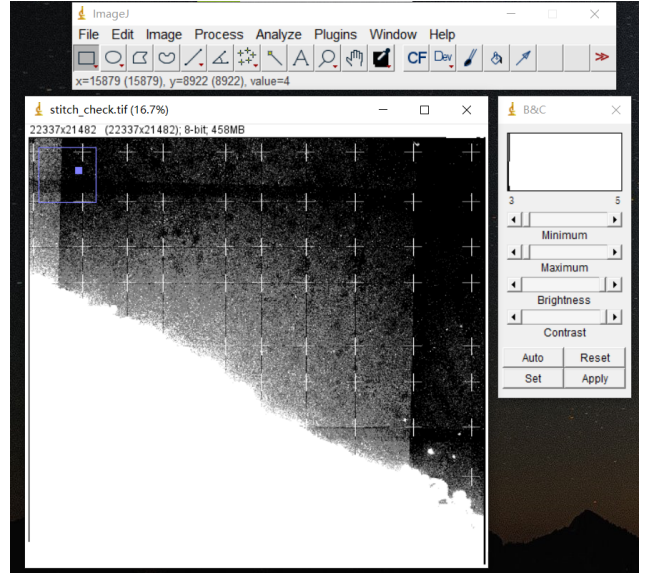
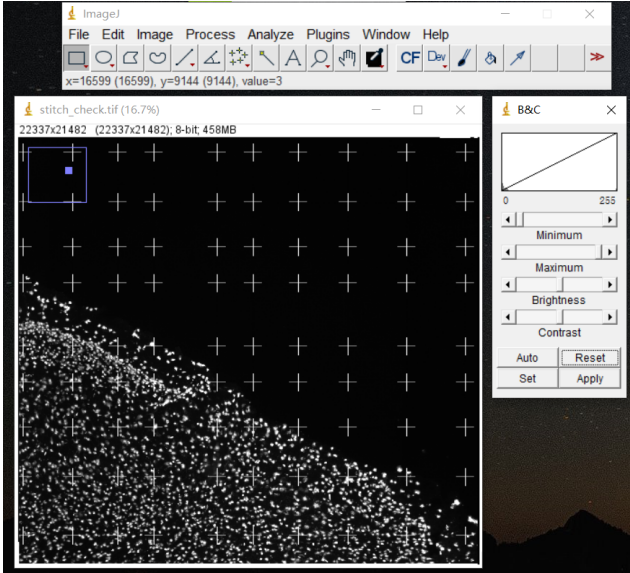


Part of the merged image of microscopy image `ssDNA_SS200000135TL_D1_regist.tif` and cell segmentation mask file `ssDNA_SS200000135TL_D1_mask.tif` to check cell segmentation performance.

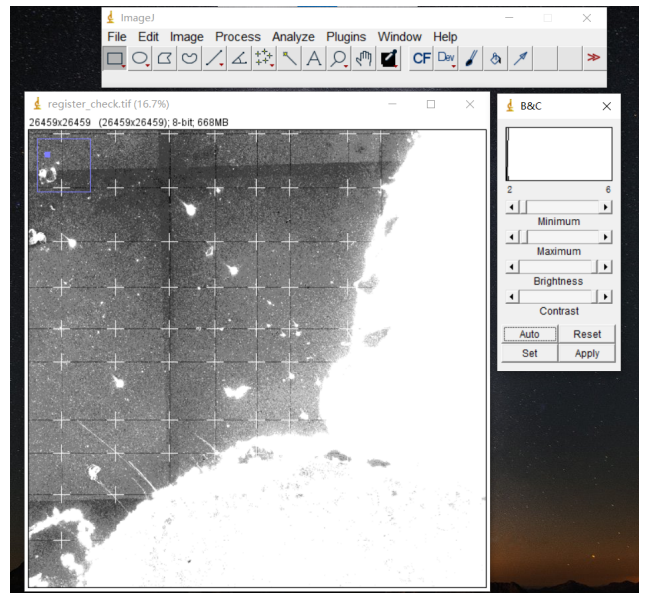
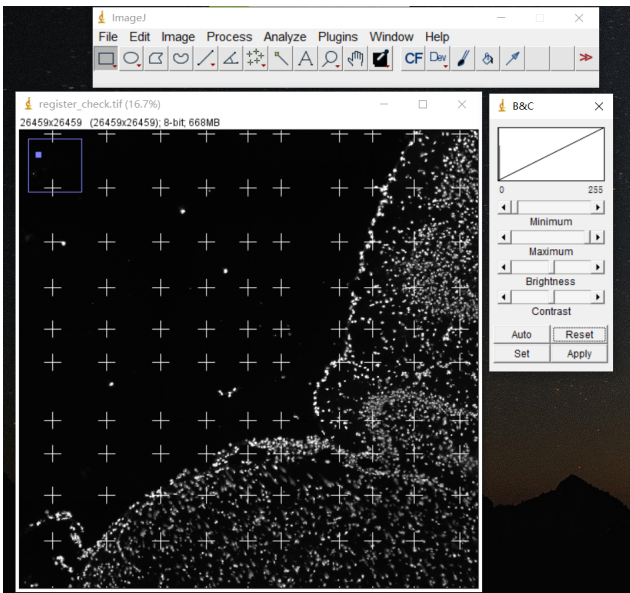


### 4.4.4 ImageTools overlay

Stack stitching template onto the `ssDNA_fov_stitched_transformed.tif` to check the result of stitching.



Stack registration template onto the `ssDNA_SS200000135TL_D1_register.tif` to check the result of registration.



## 4.5 tissueCut

### 4.5.1 Statistical Report for Tissue Covered Region

```

$ cat /path/to/output/05.tissuecut/tissuecut.stat
# Tissue Statistic Analysis with Stain Image
Contour_area: 87086375
Number_of_DNB_under_tissue: 36521212
Ratio: 41.94%
Total_gene_type: 24289
MID_counts: 89679129
Fraction_MID_in_spots_under_tissue: 82.76%
Reads_under_tissue: 709807297
Fraction_reads_in_spots_under_tissue: 83.99%

binSize=1
Mean_reads_per_spot: 15.10
Median_reads_per_spot: 9.00
Mean_gene_type_per_spot: 1.71
Median_gene_type_per_spot: 1
Mean_Umi_per_spot: 2.46
Median_Umi_per_spot: 2

binSize=20
Mean_reads_per_spot: 3241.11
Median_reads_per_spot: 2782.00
Mean_gene_type_per_spot: 241.08
Median_gene_type_per_spot: 227
Mean_Umi_per_spot: 409.56
Median_Umi_per_spot: 370

binSize=50
Mean_reads_per_spot: 20054.45
Median_reads_per_spot: 18285.00
Mean_gene_type_per_spot: 1165.56
Median_gene_type_per_spot: 1133
Mean_Umi_per_spot: 2534.10
Median_Umi_per_spot: 2346

binSize=100
Mean_reads_per_spot: 78867.48
Median_reads_per_spot: 72545.00
Mean_gene_type_per_spot: 3110.83
Median_gene_type_per_spot: 3117
Mean_Umi_per_spot: 9964.35
Median_Umi_per_spot: 9205

binSize=150
Mean_reads_per_spot: 174614.34
Median_reads_per_spot: 162073.00
Mean_gene_type_per_spot: 4926.51
Median_gene_type_per_spot: 5065
Mean_Umi_per_spot: 22066.71
Median_Umi_per_spot: 20430

binSize=200
Mean_reads_per_spot: 305687.91
Median_reads_per_spot: 285723.00
Mean_gene_type_per_spot: 6424.38
Median_gene_type_per_spot: 6747
Mean_Umi_per_spot: 38621.50
Median_Umi_per_spot: 36060
```

## 4.5.2 Example of Gene Expression Matrix for Tissue Covered Region

```

$ h5dump -n /path/to/output/05.tissuecut/SS200000135TL_D1.tissue.gef
HDF5 "/path/to/output/05.tissuecut/SS200000135TL_D1.tissue.gef" {
FILE_CONTENTS {
  group      /
  group      /geneExp
  group      /geneExp/bin1
  dataset    /geneExp/bin1/exon
  dataset    /geneExp/bin1/expression
  dataset    /geneExp/bin1/gene
}
}

$ h5dump -d /geneExp/bin1/expression /path/to/output/05.tissuecut/SS200000135TL_D1.tissue.gef | head -15
HDF5 "/path/to/output/05.tissuecut/SS200000135TL_D1.tissue.gef" {
DATASET "/geneExp/bin1/expression" {
  DATATYPE  H5T_COMPOUND {
    H5T_STD_U32LE "x";
    H5T_STD_U32LE "y";
    H5T_STD_U8LE  "count";
  }
  DATASPACE SIMPLE { ( 62542665 ) / ( 62542665 ) }
  DATA {
    (0): {
      6148,
      10906,
      1
    },
    (1): {

```

```

$ h5dump -d /geneExp/bin1/gene /path/to/output/05.tissuecut/SS200000135TL_D1.tissue.gef | head -20
HDF5 "/path/to/output/05.tissuecut/SS200000135TL_D1.tissue.gef" {
DATASET "/geneExp/bin1/gene" {
  DATATYPE  H5T_COMPOUND {
    H5T_STRING {
      STRSIZE 64;
      STRPAD  H5T_STR_NULLTERM;
      CSET   H5T_CSET_ASCII;
      CTYPE  H5T_C_S1;
    } "gene";
    H5T_STD_U32LE "offset";
    H5T_STD_U32LE "count";
  }
  DATASPACE SIMPLE { ( 24289 ) / ( 24289 ) }
  DATA {
    (0): {
      "0610005C13Rik",
      0,
      24
    },
    (1): {

```

### 4.5.3 Example of Gene Expression Matrix for complete GEF

```

$ h5dump -n /path/to/output/02.count/SS200000135TL_D1.gef
HDF5 "/path/to/output/05.tissuecut/SS200000135TL_D1.gef" {
FILE_CONTENTS {
  group      /
  group      /geneExp
  group      /geneExp/bin1
  dataset    /geneExp/bin1/exon
  dataset    /geneExp/bin1/expression
  dataset    /geneExp/bin1/gene
  group      /geneExp/bin10
  dataset    /geneExp/bin10/exon
  dataset    /geneExp/bin10/expression
  dataset    /geneExp/bin10/gene
  group      /geneExp/bin100
  dataset    /geneExp/bin100/exon
  dataset    /geneExp/bin100/expression
  dataset    /geneExp/bin100/gene
  group      /geneExp/bin20
  dataset    /geneExp/bin20/exon
  dataset    /geneExp/bin20/expression
  dataset    /geneExp/bin20/gene
  group      /geneExp/bin200
  dataset    /geneExp/bin200/exon
  dataset    /geneExp/bin200/expression
  dataset    /geneExp/bin200/gene
  group      /geneExp/bin50
  dataset    /geneExp/bin50/exon
  dataset    /geneExp/bin50/expression
  dataset    /geneExp/bin50/gene
  group      /geneExp/bin500
  dataset    /geneExp/bin500/exon
  dataset    /geneExp/bin500/expression
  dataset    /geneExp/bin500/gene
  group      /stat
  dataset    /stat/gene
  group      /wholeExp
  dataset    /wholeExp/bin1
  dataset    /wholeExp/bin10
  dataset    /wholeExp/bin100
  dataset    /wholeExp/bin20
  dataset    /wholeExp/bin200
  dataset    /wholeExp/bin50
  dataset    /wholeExp/bin500
  group      /wholeExpExon
  dataset    /wholeExpExon/bin1
  dataset    /wholeExpExon/bin10
  dataset    /wholeExpExon/bin100
  dataset    /wholeExpExon/bin20
  dataset    /wholeExpExon/bin200
  dataset    /wholeExpExon/bin50
  dataset    /wholeExpExon/bin500
}
}

```

```

$ h5dump -d /stat/gene /path/to/output/02.count/SS200000135TL_D1.gef | head -20
HDF5 "/path/to/output/05.tissuecut/SS200000135TL_D1.gef" {
DATASET "/stat/gene" {
  DATATYPE H5T_COMPOUND {
    H5T_STRING {
      STRSIZE 64;
      STRPAD H5T_STR_NULLTERM;
      CSET H5T_CSET_ASCII;
      CTYPE H5T_C_S1;
    } "gene";
    H5T_STD_U32LE "MIDcount";
    H5T_IEEE_F32LE "E10";
  }
  DATASPACE SIMPLE { ( 24670 ) / ( 24670 ) }
  DATA {
    (0): {
      "Gm42418",
      5860952,
      60.1028
    },
    (1): {

```

## 4.6 cellCut

### 4.6.1 Example of Gene Expression Matrix for Cell Bins

```

$ h5dump -n /path/to/output/051.cellcut/SS200000135TL_D1.cellbin.gef
HDF5 "/path/to/output/051.cellcut/SS200000135TL_D1.cellbin.gef" {
FILE_CONTENTS {
  group      /
  group      /cellBin
  dataset    /cellBin/blockIndex
  dataset    /cellBin/blockSize
  dataset    /cellBin/cell
  dataset    /cellBin/cellBorder
  dataset    /cellBin/cellExon
  dataset    /cellBin/cellExp
  dataset    /cellBin/cellExpExon
  dataset    /cellBin/cellTypeList
  dataset    /cellBin/gene
  dataset    /cellBin/geneExon
  dataset    /cellBin/geneExp
  dataset    /cellBin/geneExpExon
}
}

```



## 4.7 saturation

### 4.7.1 Example of Sequence Saturation File

```

$ cat /path/to/output/07.saturation/sequence_saturation.tsv
sample bar_x bar_y1 bar_y2 bar_umi bin_x bin_y1 bin_y2 bin_umi
0.05 26687198 3030 7041 1 20002730 26687198
0.273744 53374396 0.389862 1 32565765 53374396
0.409615 106748792 0.542456 1 48842313 106748792
0.557064 160123200 0.625182 1 60017028 160123200
0.636707 213497584 0.677501 1 68852648 213497584
0.6871 266871984 0.713814 1 76374913 266871984
0.722081 320246400 0.740741 1 83026752 320246400
0.748035 373620768 0.761599 1 89071589 373620768
0.768155 426995168 0.778242 1 94689701 426995168
0.784222 480369568 0.79188 1 99974373 480369568
0.797395 533743961 0.803326 1 104973406 533743961
0.808462 6718 36641

```

## 4.8 report

### 4.8.1 Example of Statistical Summary Report

```

$ head /path/to/output/08.report/SS200000135TL_D1.statistics.json
{
  "version": "version_v2",
  "1.Filter_and_Map": {
    "1.1.Adapter_Filter": [
      {
        "Sample_id": "E100026571_L01_trim_read_1",
        "getCIDPositionMap_uniqCIDTypes": "645784920",
        "total_reads": "1002214171",
        "CID_withN_reads": "8031 (0.00 %)",
        "mapped_reads": "845113606 (84.32 %)",

```



## 4.8.2 HTML Report

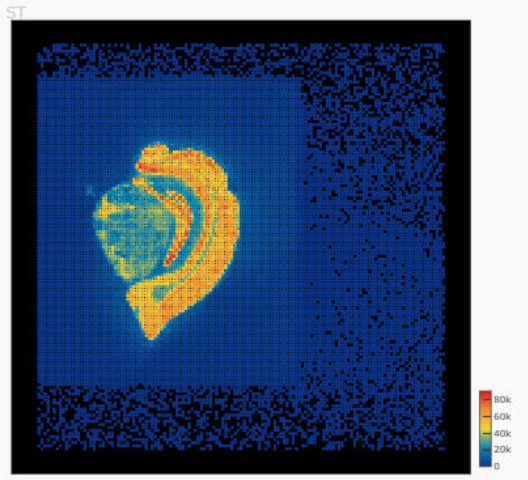


SS200000135TL\_D1

### I Alerts

Summary Square Bin Cell Bin Image

### I Spatial Gene Expression Distribution ssDNA



show image  show expression

Tissue Plot with Spot (bin200) Colored by MID Count

**1,002,214,171**

Total Reads

**38,621**

Mean MID per Bin200

**6,424**

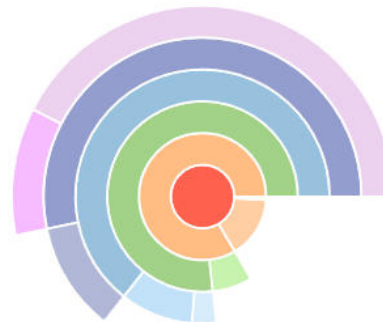
Mean Gene Type per Bin200

**108,363,128**

Unique Reads

### I Key Metrics ?

|                          |               |       |
|--------------------------|---------------|-------|
| Total Reads              | 1,002,214,171 | 100%  |
| Valid CID Reads          | 836,662,448   | 83.5% |
| Clean Reads              | 769,052,654   | 91.9% |
| Uniquely Mapped Reads    | 645,319,822   | 83.9% |
| Transcriptome            | 533,743,961   | 82.7% |
| Unique Reads             | 108,363,128   | 20.3% |
| Sequencing Saturation    | 425,380,833   | 79.7% |
| Unannotated Reads        | 111,575,861   | 17.3% |
| Multi-Mapped Reads       | 93,161,462    | 12.1% |
| Unmapped Reads           | 30,571,370    | 4.0%  |
| Non-Relevant Short Reads | 67,609,794    | 8.1%  |
| Invalid CID Reads        | 157,100,565   | 15.7% |
| Discarded MID Reads      | 8,451,158     | 0.8%  |



### I Information

|           |                            |        |       |
|-----------|----------------------------|--------|-------|
| Species   | mouse                      | Tissue | brain |
| Reference | mouse                      |        |       |
| FASTQ     | E100026571_L01_trim_read_1 |        |       |



### I Sequencing ?

|             |               |
|-------------|---------------|
| Total Reads | 1,002,214,171 |
| Total Q30   | 91.08%        |
| CID Q30     | 91.74%        |
| MID Q30     | 89.45%        |

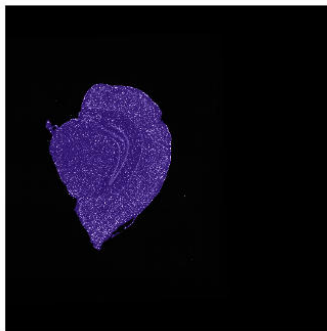
### I RNA Mapping ?

|                       |             |
|-----------------------|-------------|
| Clean Reads           | 769,052,654 |
| Uniquely Mapped Reads | 645,319,822 |
| Multi-Mapped Reads    | 93,161,462  |
| Unmapped Reads        | 30,571,370  |

### I Annotation ?

|               |             |
|---------------|-------------|
| Exonic        | 484,480,416 |
| Intronic      | 49,263,545  |
| Intergenic    | 111,575,861 |
| Transcriptome | 533,743,961 |
| Antisense     | 110,185,975 |

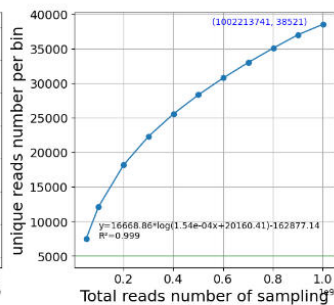
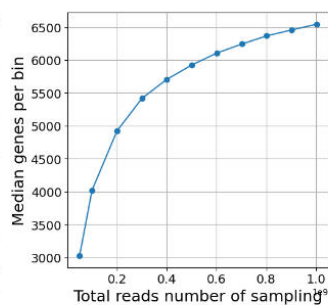
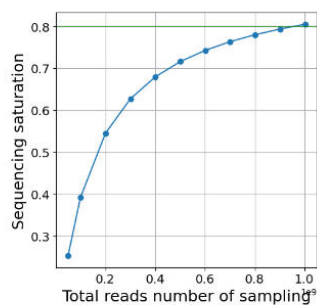
### I Tissue Segmentation



### I Tissue ?

|                                      |             |
|--------------------------------------|-------------|
| DNB Under Tissue                     | 87,086,375  |
| mRNA-Captured DNBS Under Tissue      | 36,521,212  |
| Genes Under Tissue                   | 24,289      |
| Number of MID Under Tissue Coverage  | 89,679,129  |
| Fraction MID in Spots Under Tissue   | 82.76%      |
| Reads Under Tissue                   | 709,807,297 |
| Fraction Reads in Spots Under Tissue | 83.99%      |

### I Sequencing Saturation ?



时空组学  
STOmics Analytical Report

SS200000135TL\_D1

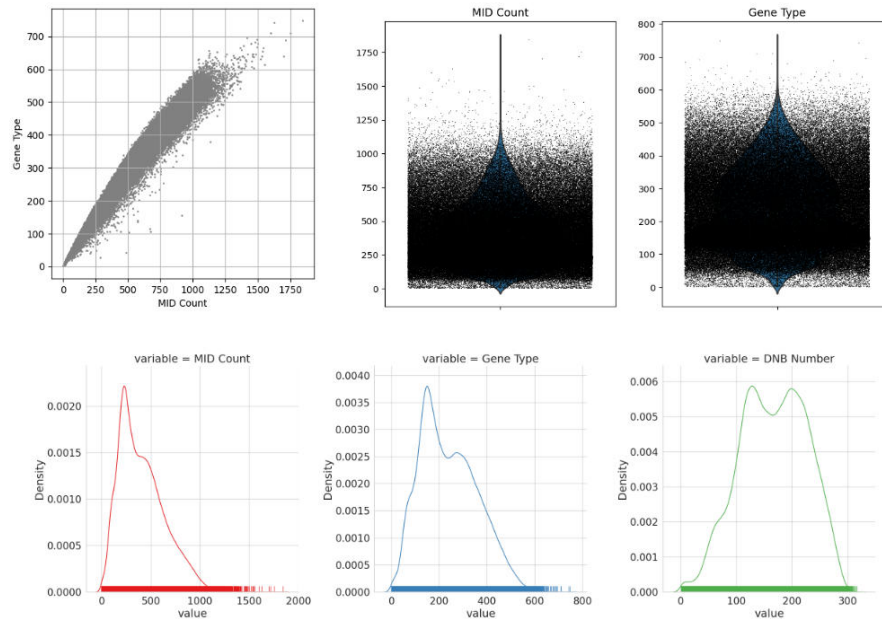
I Alerts

Summary Square Bin Cell Bin Image

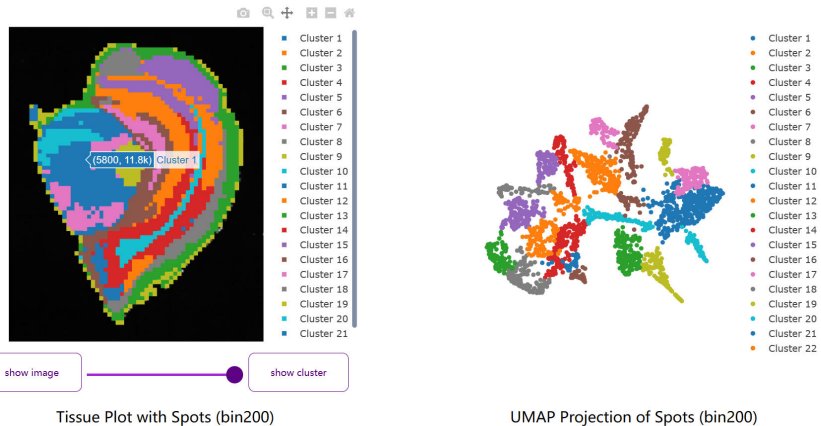
I Tissue Square Bin Statistics ?

| Bin Size | Mean Reads (per bin) | Median Reads (per bin) | Mean Gene Type (per bin) | Median Gene Type (per bin) | Mean MID (per bin) | Median MID (per bin) |
|----------|----------------------|------------------------|--------------------------|----------------------------|--------------------|----------------------|
| 1        | 15.10                | 9                      | 1.71                     | 1                          | 2.46               | 2                    |
| 20       | 3,241                | 2,782                  | 241                      | 227                        | 409                | 370                  |
| 50       | 20,054               | 18,285                 | 1,165                    | 1,133                      | 2,534              | 2,346                |
| 100      | 78,867               | 72,545                 | 3,110                    | 3,117                      | 9,964              | 9,205                |
| 150      | 174,614              | 162,073                | 4,926                    | 5,065                      | 22,066             | 20,430               |
| 200      | 305,687              | 285,723                | 6,424                    | 6,747                      | 38,621             | 36,060               |

I Bin 20 ?



I Clustering ?



SS200000135TL\_D1

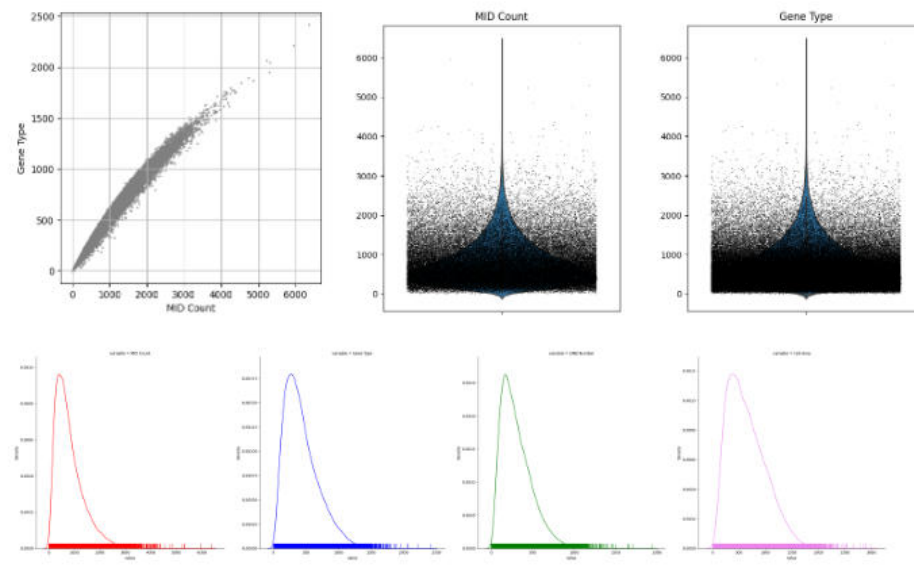
I Alerts

Summary Square Bin Cell Bin Image

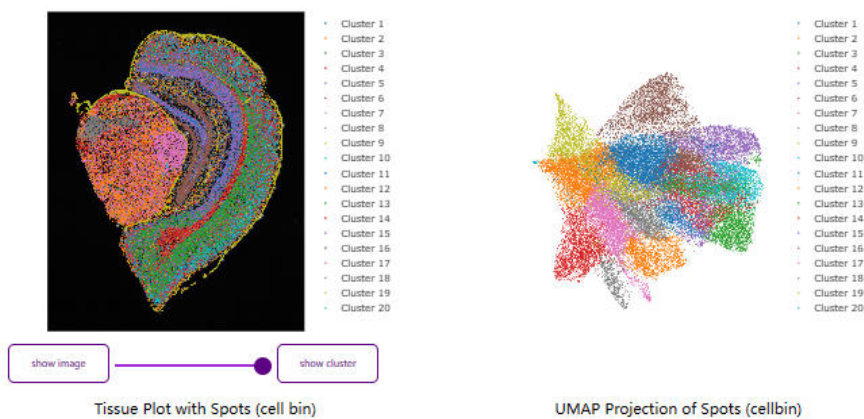
I Cell Bin Statistics ⓘ

|                |        |                  |     |
|----------------|--------|------------------|-----|
| Cell Count     | 76,873 |                  |     |
| Mean Cell Area | 648    | Median Cell Area | 586 |
| Mean DNB Count | 305    | Median DNB Count | 268 |
| Mean Gene Type | 431    | Median Gene Type | 378 |
| Mean MID       | 803    | Median MID       | 666 |

I Cell Bin ⓘ



I Clustering ⓘ



SS200000135TL\_D1

I Alerts

Summary Square Bin Cell Bin Image

I Image Information

|                    |   |
|--------------------|---|
| Manufacture        | motic                                     |
| Model              | MoticamS6 M                               |
| Scan Time          | 2021-11-25 12:26:19                       |
| Overlap            | 0.1                                       |
| Exposure Time (ms) | 19.009                                    |
| Scan Rows          | 13  |
| Scan Columns       | 9   |
| FOV Height         | 2,039                                     |
| FOV Width          | 3,064                                     |
| Stain Type         | ssDNA                                     |
| Stitched Image     | No  |
| Image Name         | SS200000135TL_D1_SC_20231031_203934_3.0.0 |

I QC

|                           |       |
|---------------------------|-------|
| ImageQC Version           | 3.0.0 |
| QC Pass                   | Pass  |
| Track Line Score          | 94    |
| Clarity Score             | 82    |
| Good FOV Count            | 98    |
| Total FOV Count           | 117   |
| Stitching Score           | 100   |
| Tissue Segmentation Score | 100   |
| Registration Score        | 99    |

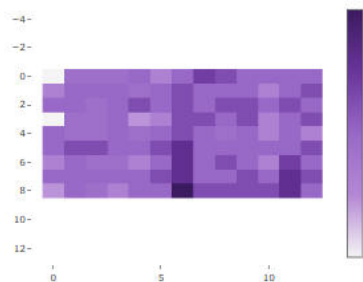
I Stitching

|                            |        |
|----------------------------|--------|
| Template Source Row No.    | 7      |
| Template Source Column No. | 7      |
| Global Height              | 24,071 |
| Global Width               | 25,128 |

I Registration

|                            |           |
|----------------------------|-----------|
| ScaleX                     | 1.156     |
| ScaleY                     | 1.153     |
| Rotation                   | -1.564    |
| Flip                       | True      |
| Image X Offset             | -3,367.76 |
| Image Y Offset             | 2.081     |
| Manual ScaleX              | -         |
| Manual ScaleY              | -         |
| Manual Rotation            | -         |
| Counter Clockwise Rotation | 90        |
| Matrix X Offset            | 0.00      |
| Matrix Y Offset            | 0.00      |
| Matrix Height              | 26,459    |
| Matrix Width               | 26,459    |

Horizontal Image Stitching Deviation Heatmap



Vertical Image Stitching Deviation Heatmap



# CHAPTER 5

TEST DATA

DEMONSTRATION

–

PROTEOMICS &

TRANSCRIPTOMICS

Users may refer to this section as a format for testing SAW process, of the files shown in this chapter as the reference in testing SAW pipelines. This chapter includes the statistics results and examples of critical files for each key step.

SN:A02677B5



“...” in the demo stands for some lines of log information that can be omitted.

## 5.1 mapping

### 5.1.1 Statistical Report for CID Mapping and Filtering

```

$ cat /path/to/output/01T.mapping/V350248064_L01_read_1.CIDMap.stat
...
        unique_CID_in_mask:      551164442
        unique_CID_in_fq:        82769358
        total_reads:             325558079
        CID_with_N_reads:        223           0.00 %
        CID_mapped_reads:        263145167      80.83 %
        CID_exactly_mapped_reads: 215239025      66.11 %
        CID_mapped_reads_with_mismatch: 47905919      14.72 %
        discarded_MID_reads:     9502982       2.92 %
        MID_with_N_reads:        62668         0.02 %
        MID_with_polyA_reads:    7625           0.00 %
        MID_with_low_quality_base_reads: 9432689      2.90 %
        reads_with_dnb:          14643107      4.50 %
        reads_with_adapter:      11536847      3.54 %
        short_reads_filtered_with_polyA: 7402775      2.27 %
        reads_with_polyA:        9930604       3.05 %
        reads_with_rRNA:         0             0.00 %
        Q10_bases_in_seq:        99.20 %
        Q20_bases_in_seq:        97.40 %
        Q30_bases_in_seq:        91.14 %
        Q10_bases_in_MID:        98.29 %
        Q20_bases_in_MID:        95.14 %
        Q30_bases_in_MID:        85.33 %
        Q10_bases_in_CID:        98.99 %
        Q20_bases_in_CID:        96.99 %
        Q30_bases_in_CID:        89.18 %

```



## 5.1.2 Statistical Report for Reference Genome Alignment

```

$ cat /path/to/output/01T.mapping/V350248064_L01_read_1.Log.final.out
...
      Number of input reads |           220059456
      Average input read length |           49
      UNIQUE READS:
      Uniquely mapped reads number |        101482044
      Uniquely mapped reads % |        46.12%
      Average mapped length |        47.22
      Number of splices: Total |        2446345
      Number of splices: Annotated (sjdb) |        2108691
      Number of splices: GT/AG |        2315419
      Number of splices: GC/AG |        24032
      Number of splices: AT/AC |        2288
      Number of splices: Non-canonical |        104606
      Mismatch rate per base, % |        1.70%
      Deletion rate per base |        0.04%
      Deletion average length |        2.88
      Insertion rate per base |        0.01%
      Insertion average length |        1.08
      MULTI-MAPPING READS:
      Number of reads mapped to multiple loci |        62340513
      % of reads mapped to multiple loci |        28.33%
      Number of reads mapped to too many loci |        3625826
      % of reads mapped to too many loci |        1.65%
      UNMAPPED READS:
      Number of reads unmapped: too many mismatches |        0
      % of reads unmapped: too many mismatches |        0.00%
      Number of reads unmapped: too short |        52344265
      % of reads unmapped: too short |        23.79%
      Number of reads unmapped: other |        266808
      % of reads unmapped: other |        0.12%
      CHIMERIC READS:
      Number of chimeric reads |        0
      % of chimeric reads |        0.00%

```

## 5.1.3 Example of BAM mapping

```

$ samtools view
/path/to/output/01T.mapping/V350248064_L01_read_1.Aligned.sortedByCoord.out.bam |
head -2
V350248064L1C002R00801053349      0      1      3000093 255      13S37M *      0
0      TTAGACCACTTGATGTGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGGTGG      BCBA>FFE96CB7;
C-;48A<9@BCC@C;BADBABDCDAD@C5.+ #4-@@      NH:i:1      HI:i:1      AS:i:34 nM:i:1
Cx:i:18567      Cy:i:12717      UR:Z:C559D
V350248064L1C003R04700229281      0      1      3000093 3      13S36M1S      *
0      0      ACTTAGCACTTGATGTGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGGTGT      B@
DDECDDDE?=E0?C;>??C@DAADDCBDDDCDDCCBCCDDDD)''&#"BNH:i:2      HI:i:1      AS:i:33 nM:i:1
Cx:i:14384      Cy:i:8392      UR:Z:72BC7

```



## 5.2 merge

### 5.2.1 Example of Mapped CID List with Reads Count File

```

$ head /path/to/output/02T.merge/A02677B5.merge.barcodeReadsCount.txt
0      4885      1
0      5494      1
0      7252      1
0      7263      1
0      7392      1
0      8929      1
0      9063      2
0      12673     7
0      12845     4
0      14828     1

```

## 5.3 count

### 5.3.1 Statistical Report for MID Filtering and Gene Annotation

```

$ cat
/path/to/output/03T.count/A02677B5.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam.summary.stat
## FILTER & DEDUPLICATION METRICS
TOTAL_READS      PASS_FILTER      ANNOTATED_READS  UNIQUE_READS      FAIL_FILTER_RATE
FAIL_ANNOTATE_RATE DUPLICATION_RATE
497134569        306509812        203236286        112788155        38.34  33.69  44.50
## ANNOTATION METRICS
TOTAL_READS      MAP      EXONIC  INTRONIC      INTERGENIC      TRANSCRIPTOME  ANTISENSE
306509812        306509812        159125426      44110860        103273526        203236286
89162163
100.0  100.0  51.9  14.4  33.7  66.3  29.1

```

### 5.3.2 Example of Annotated BAM

```

$ samtools view
/path/to/output/03T.count/A02677B5.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam |
head -2
V350248064L1C002R00801053349      0      1      3000093 255      13S37M *      0      0
TTAGACCACTTGATGTGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGGTGG      BCBA>FFE96CB7;C-;48A<9@BCC@C;
BADBABDCDAD@C5.+#4-@@      NH:i:1      HI:i:1      AS:i:34 nM:i:1      Cx:i:18567      Cy:i:12717
UR:Z:C559D      XF:i:2
V350248064L1C003R04700229281      512      1      3000093 3      13S36M1S      *      0
0      ACTTAGCACTTGATGTGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGGTGT      B@DDECDDE?=E0?C;>??C@
DAADDCBDDDCDDCCBCCDDD)'&#"BNH:i:2      HI:i:1      AS:i:33 nM:i:1      Cx:i:14384      Cy:i:8392
UR:Z:72BC7

```

### 5.3.3 Example of count Gene Expression Matrix

```

$ h5dump -n /path/to/output/03T.count/A02677B5.raw.gef
HDF5 "/path/to/output/03T.count/A02677B5.raw.gef" {
FILE_CONTENTS {
  group /
  group /geneExp
  group /geneExp/bin1
  dataset /geneExp/bin1/exon
  dataset /geneExp/bin1/expression
  dataset /geneExp/bin1/gene
}
}

$ h5dump -d /geneExp/bin1/expression /path/to/output/03T.count/A02677B5.raw.gef |
head -15
HDF5 "/path/to/output/03T.count/A02677B5.raw.gef" {
DATASET "/geneExp/bin1/expression" {
  DATATYPE H5T_COMPOUND {
    H5T_STD_U32LE "x";
    H5T_STD_U32LE "y";
    H5T_STD_U8LE "count";
  }
  DATASPACE SIMPLE { ( 97081672 ) / ( 97081672 ) }
  DATA {
    (0): {
      2630,
      8744,
      1
    },
    (1): {

```

```

$ h5dump -d /geneExp/bin1/gene /path/to/output/03T.count/A02677B5.raw.gef | head -
20
HDF5 "/path/to/output/03T.count/A02677B5.raw.gef" {
DATASET "/geneExp/bin1/gene" {
  DATATYPE H5T_COMPOUND {
    H5T_STRING {
      STRSIZE 64;
      STRPAD H5T_STR_NULLTERM;
      CSET H5T_CSET_ASCII;
      CTYPE H5T_C_S1;
    } "gene";
    H5T_STD_U32LE "offset";
    H5T_STD_U32LE "count";
  }
  DATASPACE SIMPLE { ( 27445 ) / ( 27445 ) }
  DATA {
    (0): {
      "0610005C13Rik",
      0,
      799
    },
    (1): {

```

### 5.3.4 Example of count Sampling File

```

$ head -8 /path/to/output/03T.count/A02677B5_raw_barcode_gene_exp.txt
x      y      geneIndex      MIDIndex      readCount
12053  13111  14468  282639  2
7295   9953   14469  599164  1
21748  23094  14469  477127  1
11735  13821  14470  400133  5
16697  12775  14470  411141  1
3790   22503  14470  119157  1
13954  13219  14470  413356  1

```

## 5.4 mapping-SP

### 5.4.1 Statistical Report for CID Mapping, MID filtering and PID alignment

```

$ cat /path/to/01P.mapping/A02677B5_map.stat
...
                unique_CID_in_mask:      551164442
                unique_CID_in_fq:        74809591
                total_reads:             573659235
                duplication_rate:         59.07 %
                CID_mapped_reads:         475103289      82.82 %
                CID_exactly_mapped_read:  404140856      70.45 %
                CID_mapped_reads_with_mismatch: 70962433      12.37 %
                CID_with_N_reads:         7354           0.00 %
                discarded_MID_reads:      1489710         0.26 %
                MID_with_N_reads:         4583           0.00 %
                MID_with_low_quality_base_reads: 1485127         0.26 %
                PID_mapped_reads:         427761398      90.32 %
                PID_exactly_mapped_reads:  398063274      84.05 %
                PID_mapped_reads_with_mismatch: 29698124       6.27 %
                PID_mapped_unique_reads:   175086793      36.97 %
                Q10_bases_in_CID:         99.70 %
                Q20_bases_in_CID:         97.63 %
                Q30_bases_in_CID:         87.72 %
                Q10_bases_in_MID:         99.63 %
                Q20_bases_in_MID:         97.41 %
                Q30_bases_in_MID:         88.10 %
                Q10_bases_in_ADT:         99.49 %
                Q20_bases_in_ADT:         97.49 %
                Q30_bases_in_ADT:         93.44 %

```

### 5.4.2 Example of mapping-SP Mapped CID List with Reads Count File

```

$ head /path/to/01P.mapping/A02677B5_valid_cid_reads.tsv
x      y readCount
13012  22382  2
4261   13080  2
16225  13353  14
9533   4088   4
18486  23899  3
13971  16291  9
9013   5905   1
4549   8517   3
13751  18063  23

```

### 5.4.3 Example of mapping-SP Sampling File

```

$ head /path/to/01P.mapping/A02677B5_cid_pid_mid_reads.tsv
x      y      PIDIndex      MID      readCount
232    26091  0      291549  4
2042   8307   0      611198  3
2238   3469   0      483548  1
2525   1134   0      417813  3
2616   20068  0      788601  7
3557   11877  0      416037  2
3698   10004  0      419111  1
3698   19417  0      422891  1
3698   19547  0      139153  1

```

### 5.4.4 Example of count Gene Expression Matrix

```

$ h5dump -n /path/to/01P.mapping-SP/A02677B5.protein.raw.gcf
HDF5 "/path/to/01P.mapping-SP/A02677B5.protein.raw.gcf" {
  FILE_CONTENTS {
    group /
    group /geneExp
    group /geneExp/bin1
    dataset /geneExp/bin1/expression
    dataset /geneExp/bin1/gene
  }
}

```

```

$ h5dump -d /geneExp/bin1/expression /path/to/01P.mapping-
SP/A02677B5.protein.raw.gef|head -15
HDF5 "/path/to/01P.mapping-SP/A02677B5.protein.raw.gef" {
DATASET "/geneExp/bin1/expression" {
  DATATYPE H5T_COMPOUND {
    H5T_STD_I32LE "x";
    H5T_STD_I32LE "y";
    H5T_STD_U8LE "count";
  }
  DATASPACE SIMPLE { ( 152263461 ) / ( 152263461 ) }
  DATA {
    (0): {
      11408,
      5590,
      1
    },
    (1): {
$ h5dump -d /geneExp/bin1/gene /path/to/01P.mapping-SP/A02677B5.protein.raw.gef|
head -20
HDF5 "/path/to/01P.mapping-SP/A02677B5.protein.raw.gef" {
DATASET "/geneExp/bin1/gene" {
  DATATYPE H5T_COMPOUND {
    H5T_STRING {
      STRSIZE 64;
      STRPAD H5T_STR_NULLTERM;
      CSET H5T_CSET_ASCII;
      CTYPE H5T_C_S1;
    } "gene";
    H5T_STD_U32LE "offset";
    H5T_STD_U32LE "count";
  }
  DATASPACE SIMPLE { ( 128 ) / ( 128 ) }
  DATA {
    (0): {
      "Ly49D_Ms",
      0,
      45274
    },
    (1): {

```

## 5.5 calibration

### 5.5.1 Example of Gene Expression Matrix for calibrated complete GEFs

```

$ h5dump -n /path/to/output/04.calibration/A02677B5.gef
HDF5 "/path/to/output/04.calibration/A02677B5.gef" {
FILE_CONTENTS {
  group      /
  group      /geneExp
  group      /geneExp/bin1
  dataset    /geneExp/bin1/exon
  dataset    /geneExp/bin1/expression
  dataset    /geneExp/bin1/gene

```

```

group      /geneExp/bin10
dataset    /geneExp/bin10/exon
dataset    /geneExp/bin10/expression
dataset    /geneExp/bin10/gene
group      /geneExp/bin100
dataset    /geneExp/bin100/exon
dataset    /geneExp/bin100/expression
dataset    /geneExp/bin100/gene
group      /geneExp/bin150
dataset    /geneExp/bin150/exon
dataset    /geneExp/bin150/expression
dataset    /geneExp/bin150/gene
group      /geneExp/bin20
dataset    /geneExp/bin20/exon
dataset    /geneExp/bin20/expression
dataset    /geneExp/bin20/gene
group      /geneExp/bin200
dataset    /geneExp/bin200/exon
dataset    /geneExp/bin200/expression
dataset    /geneExp/bin200/gene
group      /geneExp/bin5
dataset    /geneExp/bin5/exon
dataset    /geneExp/bin5/expression
dataset    /geneExp/bin5/gene
group      /geneExp/bin50
dataset    /geneExp/bin50/exon
dataset    /geneExp/bin50/expression
dataset    /geneExp/bin50/gene
group      /stat
dataset    /stat/gene
group      /wholeExp
dataset    /wholeExp/bin1
dataset    /wholeExp/bin10
dataset    /wholeExp/bin100
dataset    /wholeExp/bin150
dataset    /wholeExp/bin20
dataset    /wholeExp/bin200
dataset    /wholeExp/bin5
dataset    /wholeExp/bin50
group      /wholeExpExon
dataset    /wholeExpExon/bin1
dataset    /wholeExpExon/bin10
dataset    /wholeExpExon/bin100
dataset    /wholeExpExon/bin150
dataset    /wholeExpExon/bin20
dataset    /wholeExpExon/bin200
dataset    /wholeExpExon/bin5
dataset    /wholeExpExon/bin50
}
}

$ h5dump -d /stat/gene /path/to/output/04.calibration/A02677B5.gef | head -20
HDF5 "/path/to/output/04.calibration/A02677B5.gef" {
DATASET "/stat/gene" {
  DATATYPE H5T_COMPOUND {
    H5T_STRING {
      STRSIZE 64;
      STRPAD H5T_STR_NULLTERM;
      CSET H5T_CSET_ASCII;
      CTYPE H5T_C_S1;
    } "gene";
  }
}
}

```

```

H5T_STD_U32LE "MIDcount";
H5T_IEEE_F32LE "E10";
}
DATASPACE SIMPLE { ( 27445 ) / ( 27445 ) }
DATA {
(0): {
    "Gm42418",
    24328473,
    77.1551
},
(1): {

$ h5dump -n /path/to/output/04.calibration/A02677B5.protein.gef
HDF5 "/path/to/output/04.calibration/A02677B5.protein.gef" {
FILE_CONTENTS {
group      /
group      /geneExp
group      /geneExp/bin1
dataset    /geneExp/bin1/expression
dataset    /geneExp/bin1/gene
group      /geneExp/bin10
dataset    /geneExp/bin10/expression
dataset    /geneExp/bin10/gene
group      /geneExp/bin100
dataset    /geneExp/bin100/expression
dataset    /geneExp/bin100/gene
group      /geneExp/bin150
dataset    /geneExp/bin150/expression
dataset    /geneExp/bin150/gene
group      /geneExp/bin20
dataset    /geneExp/bin20/expression
dataset    /geneExp/bin20/gene
group      /geneExp/bin200
dataset    /geneExp/bin200/expression
dataset    /geneExp/bin200/gene
group      /geneExp/bin5
dataset    /geneExp/bin5/expression
dataset    /geneExp/bin5/gene
group      /geneExp/bin50
dataset    /geneExp/bin50/expression
dataset    /geneExp/bin50/gene
group      /stat
dataset    /stat/gene
group      /wholeExp
dataset    /wholeExp/bin1

```

```
dataset /wholeExp/bin10
dataset /wholeExp/bin100
dataset /wholeExp/bin150
dataset /wholeExp/bin20
dataset /wholeExp/bin200
dataset /wholeExp/bin5
dataset /wholeExp/bin50
}
}

$ h5dump -d /stat/gene /path/to/output/04.calibration/A02677B5.protein.gcf |
head -20
HDF5 "/path/to/output/04.calibration/A02677B5.protein.gcf" {
DATASET "/stat/gene" {
  DATATYPE H5T_COMPOUND {
    H5T_STRING {
      STRSIZE 64;
      STRPAD H5T_STR_NULLTERM;
      CSET H5T_CSET_ASCII;
      CTYPE H5T_C_S1;
    } "gene";
    H5T_STD_U32LE "MIDcount";
    H5T_IEEE_F32LE "E10";
  }
  DATASPACE SIMPLE { ( 128 ) / ( 128 ) }
  DATA {
    (0): {
      "CD3_Ms",
      19264971,
      73.0514
    },
    (1): {
```



## 5.6 register and imageTools

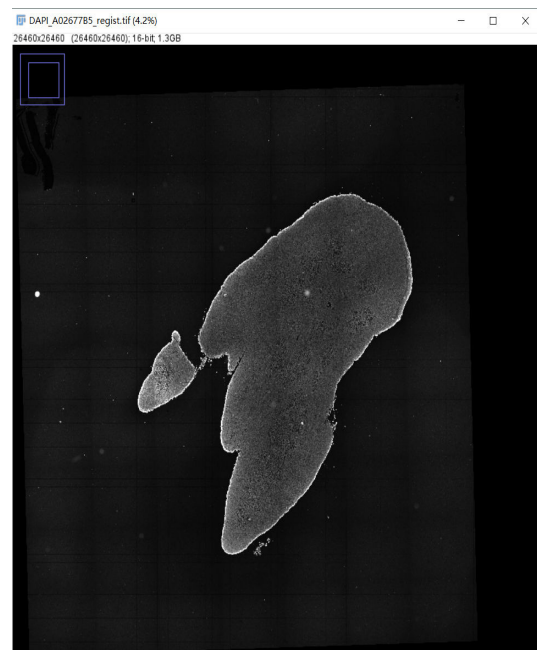
### 5.6.1 Registered image

File /path/to/output/05.register/DAPI\_fov\_stitched\_transformed.tif and /path/to/output/05.register/DAPI\_A02677B5\_regist.tif.

/path/to/output/05.register/DAP  
I\_fov\_stitched\_transformed.tif




/path/to/output/05.register/DAPI\_  
A02677B5\_regist.tif



### 5.6.2 ImageTools merge

 You can confirm tissue and cell segmentation via imageTools merge. You can check [4.4.3 imageTools merge](#) for more information.

### 5.6.3 ImageTools overlay

 You can confirm tissue and cell segmentation via imageTools merge. You can check [4.4.3 imageTools merge](#) for more information.

## 5.6.4 Image Process Record File

```

$ h5dump -n /path/to/output/05.register/A02677B5_SC_20240222_150321_3.0.3.ipr
HDF5 "/path/to/output/05.register/A02677B5_SC_20240222_150321_3.0.3.ipr" {
FILE_CONTENTS {
  group      /
  group      /DAPI
  group      /DAPI/CellSeg
  dataset    /DAPI/CellSeg/CellMask
  dataset    /DAPI/CellSeg/CellSegTrace
  group      /DAPI/ImageInfo
  dataset    /DAPI/ImageInfo/RGBScale
  group      /DAPI/QCInfo
  dataset    /DAPI/QCInfo/ClarityArr
  group      /DAPI/QCInfo/CrossPoints
  dataset    /DAPI/QCInfo/CrossPoints/0_0
  ...
  dataset    /DAPI/QCInfo/CrossPoints/8_6
  group      /DAPI/Register
  dataset    /DAPI/Register/MatrixTemplate
  group      /DAPI/Stitch
  group      /DAPI/Stitch/ScopeStitch
  dataset    /DAPI/Stitch/ScopeStitch/GlobalLoc
  dataset    /DAPI/Stitch/ScopeStitch/ScopeHorizontalJitter
  dataset    /DAPI/Stitch/ScopeStitch/ScopeJitterDiff
  dataset    /DAPI/Stitch/ScopeStitch/ScopeVerticalJitter
  group      /DAPI/Stitch/StitchEval
  dataset    /DAPI/Stitch/StitchEval/GlobalDeviation
  dataset    /DAPI/Stitch/StitchEval/StitchEvalH
  dataset    /DAPI/Stitch/StitchEval/StitchEvalV
  dataset    /DAPI/Stitch/TemplatePoint
  dataset    /DAPI/Stitch/TransformTemplate
  group      /DAPI/TissueSeg
  dataset    /DAPI/TissueSeg/TissueMask
  group      /ManualState
  dataset    /Preview
  group      /StereoResepSwitch
}
}
$ h5dump -A /path/to/output/05.register/A02677B5_SC_20240222_150321_3.0.3.ipr |
head -20
HDF5 "/path/to/output/05.register/A02677B5_SC_20240222_150321_3.0.3.ipr" {
GROUP "/" {
  ATTRIBUTE "IPRVersion" {
    DATATYPE  H5T_STRING {
      STRSIZE  H5T_VARIABLE;
      STRPAD   H5T_STR_NULLTERM;
      CSET     H5T_CSET_UTF8;
      CTYPE   H5T_C_S1;
    }
    DATASPACE  SCALAR
    DATA {
      (0): "0.2.0"
    }
  }
  GROUP "DAPI" {
    GROUP "CellSeg" {
      ATTRIBUTE "CellSegShape" {
        DATATYPE  H5T_STD_I64LE
        DATASPACE  SIMPLE { ( 2 ) / ( 2 ) }
        DATA {

```

## 5.7 tissueCut

### 5.7.1 Statistical Report for Tissue Covered Region

```
$ cat /path/to/output/06T.tissuecut/tissuecut.stat
# Tissue Statistic Analysis with Stain Image
Contour_area: 48203139
Number_of_DNB_under_tissue: 23455424
Ratio: 48.66%
Total_gene_type: 26611
MID_counts: 84007203
Fraction_MID_in_spots_under_tissue: 74.48%
Reads_under_tissue: 491750982
Fraction_reads_in_spots_under_tissue: 60.89%

binSize=1
Mean_reads_per_spot: 18.28
Median_reads_per_spot: 16.00
Mean_gene_type_per_spot: 3.02
Median_gene_type_per_spot: 3
Mean_Umi_per_spot: 3.58
Median_Umi_per_spot: 3

binSize=20
Mean_reads_per_spot: 4037.53
Median_reads_per_spot: 4161.00
Mean_gene_type_per_spot: 401.42
Median_gene_type_per_spot: 415
Mean_Umi_per_spot: 689.86
Median_Umi_per_spot: 716

binSize=50
Mean_reads_per_spot: 24815.86
Median_reads_per_spot: 25896.00
Mean_gene_type_per_spot: 1868.18
Median_gene_type_per_spot: 1963
Mean_Umi_per_spot: 4239.79
Median_Umi_per_spot: 4450

binSize=100
Mean_reads_per_spot: 96725.21
Median_reads_per_spot: 102585.00
Mean_gene_type_per_spot: 4617.22
Median_gene_type_per_spot: 4912
Mean_Umi_per_spot: 16530.34
Median_Umi_per_spot: 17600

binSize=150
Mean_reads_per_spot: 211870.31
Median_reads_per_spot: 227950.00
Mean_gene_type_per_spot: 6723.55
Median_gene_type_per_spot: 7212
Mean_Umi_per_spot: 36194.40
Median_Umi_per_spot: 38985

binSize=200
Mean_reads_per_spot: 368628.94
Median_reads_per_spot: 401073.00
Mean_gene_type_per_spot: 8267.19
Median_gene_type_per_spot: 8889
Mean_Umi_per_spot: 63021.16
Median_Umi_per_spot: 68799
```



```
$ cat /path/to/06P.tissuecut/tissuecut.stat
# Tissue Statistic Analysis with Stain Image
Contour_area: 48203139
Number_of_DNB_under_tissue: 24749871
Ratio: 51.34%
Total_protein_type: 128
MID_counts: 113480216
Fraction_MID_in_spots_under_tissue: 64.84%
Reads_under_tissue: 310743393
Fraction_reads_in_spots_under_tissue: 65.41%

binSize=1
Mean_reads_per_spot: 12.42
Median_reads_per_spot: 10.00
Mean_protein_type_per_spot: 3.85
Median_protein_type_per_spot: 3
Mean_Umi_per_spot: 4.59
Median_Umi_per_spot: 4

binSize=20
Mean_reads_per_spot: 2551.64
Median_reads_per_spot: 2541.00
Mean_protein_type_per_spot: 91.29
Median_protein_type_per_spot: 93
Mean_Umi_per_spot: 931.85
Median_Umi_per_spot: 930

binSize=50
Mean_reads_per_spot: 15683.81
Median_reads_per_spot: 15822.00
Mean_protein_type_per_spot: 119.51
Median_protein_type_per_spot: 122
Mean_Umi_per_spot: 5727.85
Median_Umi_per_spot: 5791

binSize=100
Mean_reads_per_spot: 61133.86
Median_reads_per_spot: 62505.00
Mean_protein_type_per_spot: 125.57
Median_protein_type_per_spot: 128
Mean_Umi_per_spot: 22325.44
Median_Umi_per_spot: 22867

binSize=150
Mean_reads_per_spot: 133883.41
Median_reads_per_spot: 139390.00
Mean_protein_type_per_spot: 125.91
Median_protein_type_per_spot: 128
Mean_Umi_per_spot: 48892.81
Median_Umi_per_spot: 51058

binSize=200
Mean_reads_per_spot: 233115.83
Median_reads_per_spot: 245086.00
Mean_protein_type_per_spot: 126.11
Median_protein_type_per_spot: 128
Mean_Umi_per_spot: 85131.45
Median_Umi_per_spot: 89608
```

## 5.7.2 Example of Gene Expression Matrix for Tissue Covered Region

```

$ h5dump -n /path/to/output/06T.tissuecut/A02677B5.tissue.gef
HDF5 "/path/to/output/06T.tissuecut/A02677B5.tissue.gef" {
FILE_CONTENTS {
  group      /
  group      /geneExp
  group      /geneExp/bin1
  dataset    /geneExp/bin1/exon
  dataset    /geneExp/bin1/expression
  dataset    /geneExp/bin1/gene
}
}

$ h5dump -d /geneExp/bin1/expression
/path/to/output/06T.tissuecut/A02677B5.tissue.gef | head -15
HDF5 "/path/to/output/06T.tissuecut/A02677B5.tissue.gef" {
DATASET "/geneExp/bin1/expression" {
  DATATYPE  H5T_COMPOUND {
    H5T_STD_I32LE "x";
    H5T_STD_I32LE "y";
    H5T_STD_U8LE  "count";
  }
  DATASPACE SIMPLE { ( 70724787 ) / ( 70724787 ) }
  DATA {
    (0): {
      9713,
      15266,
      1
    },
    (1): {

```

```

$ h5dump -d /geneExp/bin1/gene /path/to/output/06T.tissuecut/A02677B5.tissue.gef |
head -20
HDF5 "/path/to/output/06T.tissuecut/A02677B5.tissue.gef" {
DATASET "/geneExp/bin1/gene" {
  DATATYPE  H5T_COMPOUND {
    H5T_STRING {
      STRSIZE 64;
      STRPAD  H5T_STR_NULLTERM;
      CSET   H5T_CSET_ASCII;
      CTYPE  H5T_C_S1;
    } "gene";
    H5T_STD_U32LE "offset";
    H5T_STD_U32LE "count";
  }
  DATASPACE SIMPLE { ( 26611 ) / ( 26611 ) }
  DATA {
    (0): {
      "0610005C13Rik",
      0,
      322
    },
    (1): {

```

```

$ h5dump -n /path/to/output/06P.tissuecut/A02677B5.protein.tissue.gef
HDF5 "/path/to/06P.tissuecut/A02677B5.protein.tissue.gef" {
FILE_CONTENTS {
  group      /
  group      /geneExp
  group      /geneExp/bin1
  dataset    /geneExp/bin1/expression
  dataset    /geneExp/bin1/gene
}
}

$ h5dump -d /geneExp/bin1/expression
/path/to/output/06P.tissuecut/A02677B5.protein.tissue.gef | head -15
HDF5 "/path/to/06P.tissuecut/A02677B5.protein.tissue.gef" {
DATASET "/geneExp/bin1/expression" {
  DATATYPE  H5T_COMPOUND {
    H5T_STD_I32LE "x";
    H5T_STD_I32LE "y";
    H5T_STD_U8LE  "count";
  }
  DATASPACE SIMPLE { ( 95273207 ) / ( 95273207 ) }
  DATA {
    (0): {
      11411,
      14924,
      1
    },
    (1): {
$ h5dump -d /geneExp/bin1/gene
/path/to/output/06P.tissuecut/A02677B5.protein.tissue.gef | head -20
HDF5 "/path/to/output/06P.tissuecut/A02677B5.protein.tissue.gef" {
DATASET "/geneExp/bin1/gene" {
  DATATYPE  H5T_COMPOUND {
    H5T_STRING {
      STRSIZE 64;
      STRPAD  H5T_STR_NULLTERM;
      CSET   H5T_CSET_ASCII;
      CTYPE  H5T_C_S1;
    } "gene";
    H5T_STD_U32LE "offset";
    H5T_STD_U32LE "count";
  }
  DATASPACE SIMPLE { ( 128 ) / ( 128 ) }
  DATA {
    (0): {
      "Ly49D_Ms",
      0,
      16828
    },
    (1): {

```

## 5.8 cellCut

### 5.8.1 Example of Gene Expression Matrix for Cell Bins

```
$ h5dump -n /path/to/output/061T.cellcut/A02677B5.cellbin.gef HDF5 "  
/path/to/output/061T.cellcut/A02677B5.cellbin.gef" {  
FILE_CONTENTS {  
  group      /  
  group      /cellBin  
  dataset    /cellBin/blockIndex  
  dataset    /cellBin/blockSize  
  dataset    /cellBin/cell  
  dataset    /cellBin/cellBorder  
  dataset    /cellBin/cellExon  
  dataset    /cellBin/cellExp  
  dataset    /cellBin/cellExpExon  
  dataset    /cellBin/cellTypeList  
  dataset    /cellBin/gene  
  dataset    /cellBin/geneExon  
  dataset    /cellBin/geneExp  
  dataset    /cellBin/geneExpExon  
}  
}
```

```
$ h5dump -n /path/to/output/061P.cellcut/A02677B5.protein.cellbin.gef  
HDF5 "/path/to/061P.cellcut/A02677B5.protein.cellbin.gef" {  
FILE_CONTENTS {  
  group      /  
  group      /cellBin  
  dataset    /cellBin/blockIndex  
  dataset    /cellBin/blockSize  
  dataset    /cellBin/cell  
  dataset    /cellBin/cellBorder  
  dataset    /cellBin/cellExp  
  dataset    /cellBin/cellTypeList  
  dataset    /cellBin/gene  
  dataset    /cellBin/geneExp  
}  
}
```

## 5.9 saturation

### 5.9.1 Example of Sequence Saturation File

```
$ cat /path/to/output/08T.saturation/sequence_saturation.tsv
sample bar_x bar_y1 bar_y2 bar_umi bin_x bin_y1 bin_y2 bin_umi
0.05 10161815 0.0526077 1 9627224 10161815 0.0686479 2668 5671
0.1 20323630 0.0976586 1 18338852 20323630 0.116931 4021 10804
0.2 40647260 0.172483 1 33636299 40647260 0.194497 5585 19816
0.3 60970888 0.232096 2 46819785 60970888 0.254876 6499 27583
0.4 81294520 0.281427 2 58416019 81294520 0.30439 7118 34415
0.5 101618144 0.322769 2 68818922 101618144 0.345701 7570 40544
0.6 121941776 0.358552 2 78219368 121941776 0.381207 7926 46083
0.7 142265392 0.389758 2 86816303 142265392 0.412077 8228 51147
0.8 162589040 0.417262 2 94746848 162589040 0.439195 8447 55820
0.9 182912656 0.441618 2 102135187 182912656 0.463109 8659 60173
1 203236286 0.46353 3 109030103 203236286 0.484589 8817 63276
```

```
$ cat /path/to/output/08P.saturation/sequence_saturation.tsv
sample bar_x bar_y1 bar_y2 bar_umi bin_x bin_y1 bin_y2 bin_umi
0.05 21388070 0.0631674 1 20037040 21388070 0.0888825 126 10218
0.1 42776140 0.120048 1 37640944 42776140 0.150368 128 19197
0.2 85552280 0.217101 2 66978781 85552280 0.249677 128 34159
0.3 128328424 0.296905 2 90227130 128328424 0.32905 128 46016
0.4 171104560 0.363266 2 108948120 171104560 0.394026 128 55564
0.5 213880704 0.418843 3 124298217 213880704 0.447933 128 63392
0.6 256656848 0.466254 3 136989686 256656848 0.493682 128 69865
0.7 299432992 0.506823 3 147673538 299432992 0.532617 128 75314
0.8 342209120 0.541944 3 156750875 342209120 0.566236 128 79943
0.9 384985248 0.572588 3 164547428 384985248 0.595516 128 83919
1 427761398 0.59948 3 171326815 427761398 0.62115 128 86073
```

## 5.10 report-PT

### 5.10.1 Example of Statistical Summary Report

```
$ head /path/to/10.report/A02677B5.statistics.json
{
  "version": "version_v2",
  "1.Filter_and_Map": {
    "1.1.Adapter_Filter": [
      {
        "Sample_id": "V350248064_L03_read_1",
        "getCIDPositionMap_uniqCIDTypes": "551164442",
        "total_reads": "336354182",
        "CID_withN_reads": "523 (0.00 %)",
        "mapped_reads": "269471919 (80.12 %)",
```



### 5.10.2 HTML Report

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STOmics
Analytical Report

A02677B5

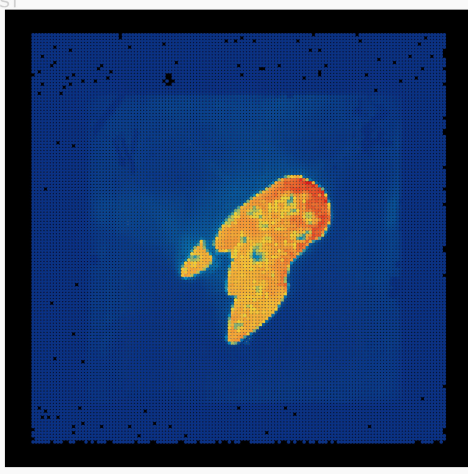
#### Alerts ⬆

Summary
Square Bin
Cell Bin
Image
Analysis

Gene
Gene
Gene

Protein
Protein
Protein

#### Spatial Gene Expression Distribution ? DAPI



**1,001,766,720**  
Total Reads

---

**63,021**  
Mean MID per Bin200

---

**8,267**  
Mean Gene per Bin200

---

**112,788,155**  
Unique Reads

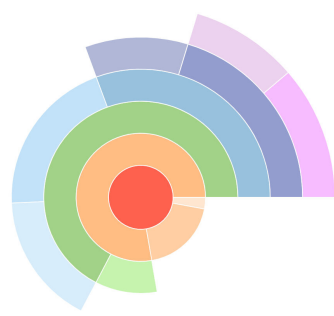
show image

show expression

Tissue Plot with Spot (bin200) Colored by MID Count

#### Key Metrics ?

|                          |               |       |
|--------------------------|---------------|-------|
| Total Reads              | 1,001,766,720 | 100%  |
| Valid CID Reads          | 778,612,149   | 77.7% |
| Clean Reads              | 674,771,804   | 86.7% |
| Uniquely Mapped Reads    | 306,509,812   | 45.4% |
| Annotated Reads          | 203,236,286   | 66.3% |
| Unique Reads             | 112,788,155   | 55.5% |
| Sequencing Saturation    | 90,448,131    | 44.5% |
| Unannotated Reads        | 103,273,526   | 33.7% |
| Multi-Mapped Reads       | 201,558,434   | 29.9% |
| Unmapped Reads           | 166,703,558   | 24.7% |
| Non-Relevant Short Reads | 103,840,345   | 13.3% |
| Invalid CID Reads        | 194,161,111   | 19.4% |
| Discarded MID Reads      | 28,993,460    | 2.9%  |



#### Information

|           |  |        |                       |
|-----------|--|--------|-----------------------|
| Species   | mouse  | Tissue | thymus                |
| Reference | mouse  |        |                       |
| FASTQ     | V350248064_L03_read_1<br>V350248064_L01_read_1 |        | V350248064_L02_read_1 |



### I Sequencing ?

|             |               |
|-------------|---------------|
| Total Reads | 1,001,766,720 |
| Total Q30   | 91.63%        |
| CID Q30     | 89.69%        |
| MID Q30     | 85.77%        |

### I RNA Mapping ?

|                       |             |
|-----------------------|-------------|
| Clean Reads           | 674,771,804 |
| Uniquely Mapped Reads | 306,509,812 |
| Multi-Mapped Reads    | 201,558,434 |
| Unmapped Reads        | 166,703,558 |

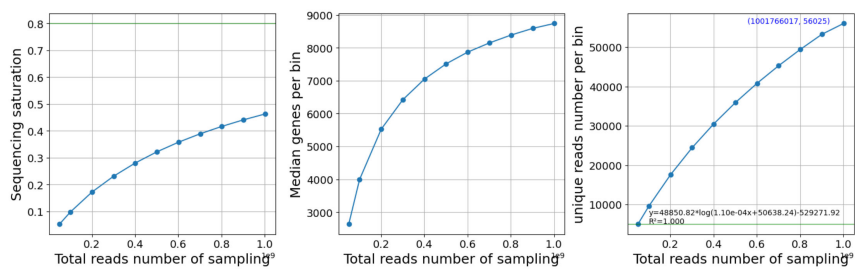
### I Annotation ?

|               |             |
|---------------|-------------|
| Exonic        | 159,125,426 |
| Intronic      | 44,110,860  |
| Intergenic    | 103,273,526 |
| Transcriptome | 203,236,286 |
| Antisense     | 89,162,163  |

### I Tissue ?

|                                      |             |
|--------------------------------------|-------------|
| DNB Under Tissue                     | 48,203,139  |
| mRNA-Captured DNBS Under Tissue      | 23,455,424  |
| Genes Under Tissue                   | 26,611      |
| Number of MID Under Tissue Coverage  | 84,007,203  |
| Fraction MID in Spots Under Tissue   | 74.48%      |
| Reads Under Tissue                   | 491,750,982 |
| Fraction Reads in Spots Under Tissue | 60.89%      |

### I Sequencing Saturation ?



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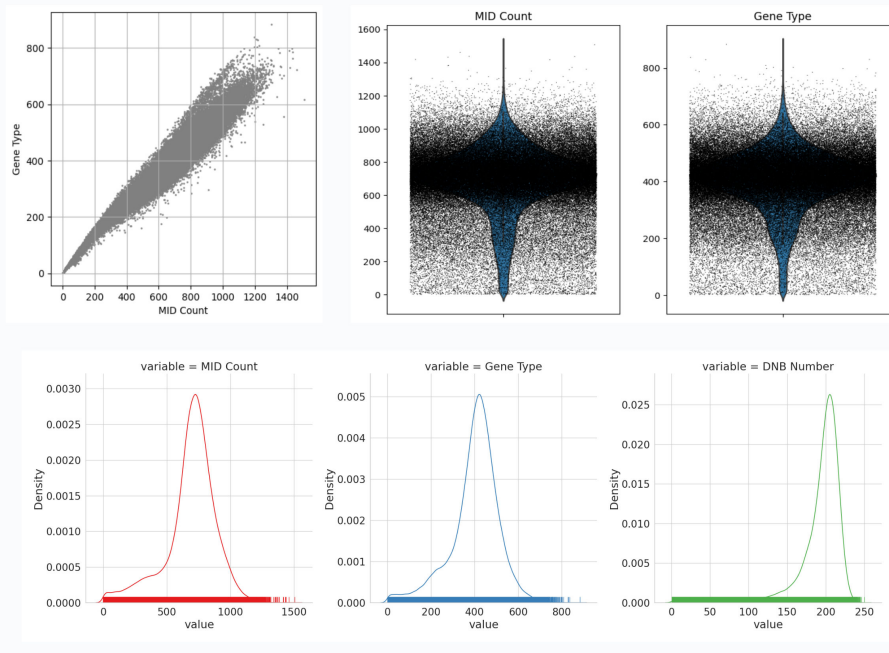
Alerts

- Summary
  - Gene
  - Protein
- Square Bin
  - Gene
  - Protein
- Cell Bin
  - Gene
  - Protein
- Image
- Analysis

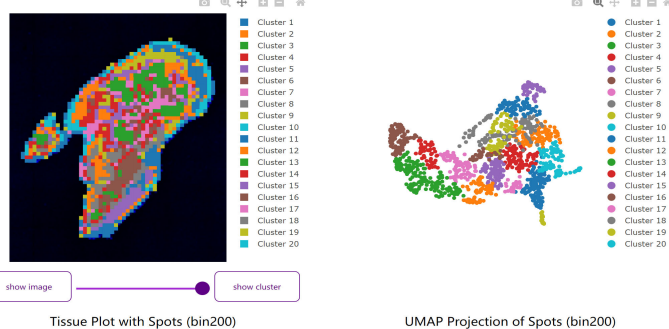
Tissue Square Bin Statistics

| Bin Size | Mean Reads (per bin) | Median Reads (per bin) | Mean Gene Type (per bin) | Median Gene Type (per bin) | Mean MID (per bin) | Median MID (per bin) |
|----------|----------------------|------------------------|--------------------------|----------------------------|--------------------|----------------------|
| 1        | 18.28                | 16                     | 3.02                     | 3                          | 3.58               | 3                    |
| 20       | 4,037                | 4,161                  | 401                      | 415                        | 689                | 716                  |
| 50       | 24,815               | 25,896                 | 1,868                    | 1,963                      | 4,239              | 4,450                |
| 100      | 96,725               | 102,585                | 4,617                    | 4,912                      | 16,530             | 17,600               |
| 150      | 211,870              | 227,950                | 6,723                    | 7,212                      | 36,194             | 38,985               |
| 200      | 368,628              | 401,073                | 8,267                    | 8,889                      | 63,021             | 68,799               |

Bin 20



Clustering



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A02677B5

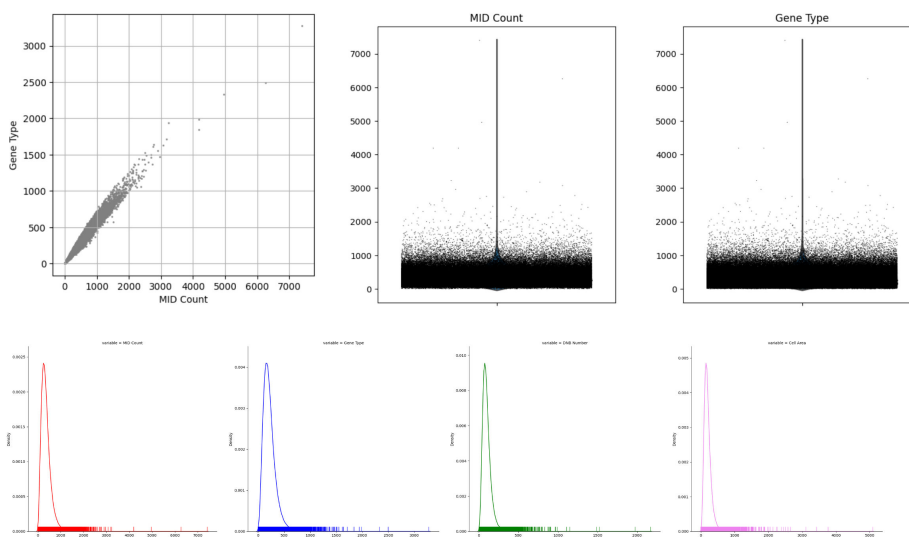
Alerts

- Summary
  - Gene
  - Protein
- Square Bin
  - Gene
  - Protein
- Cell Bin**
  - Gene
  - Protein
- Image
- Analysis

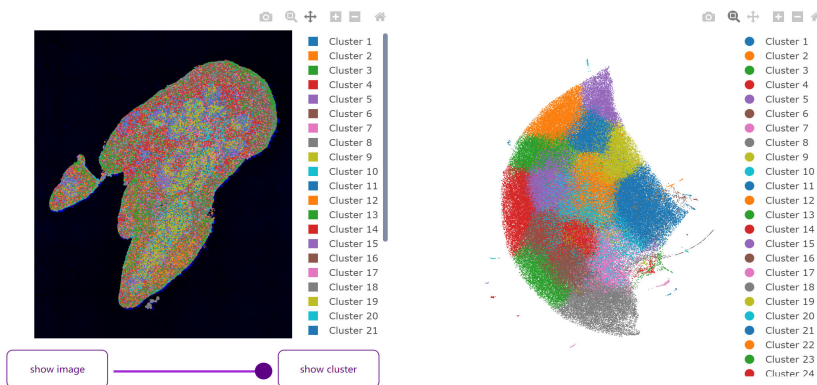
Cell Bin Statistics

|                |         |                  |     |
|----------------|---------|------------------|-----|
| Cell Count     | 198,566 |                  |     |
| Mean Cell Area | 203     | Median Cell Area | 183 |
| Mean DNB Count | 99.52   | Median DNB Count | 90  |
| Mean Gene Type | 217     | Median Gene Type | 196 |
| Mean MID       | 357     | Median MID       | 318 |

Cell Bin



Clustering



Tissue Plot with Spots (cell bin)

UMAP Projection of Spots (cellbin)



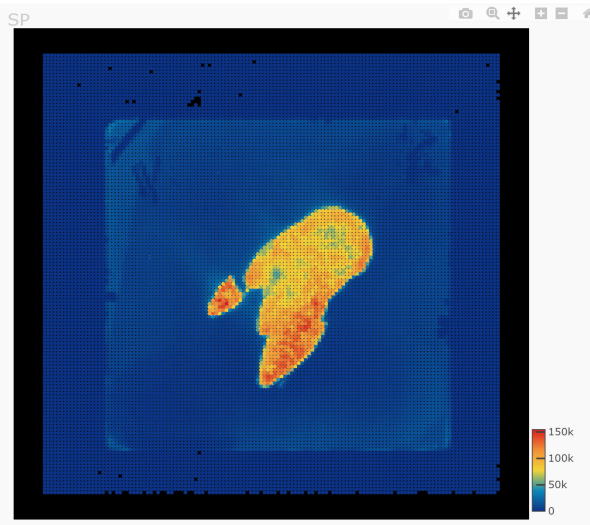
时空组学 STOmics Analytical Report

A02677B5

Alerts

- Summary
  - Gene
  - Protein
- Square Bin
  - Gene
  - Protein
- Cell Bin
  - Gene
  - Protein
- Image
- Analysis

Spatial Protein Expression Distribution



show image  show expression

Tissue Plot with Spot (bin200) Colored by MID Count

**573,659,235**

Total Reads

**473,613,579**

Valid CID Reads

**427,761,398**

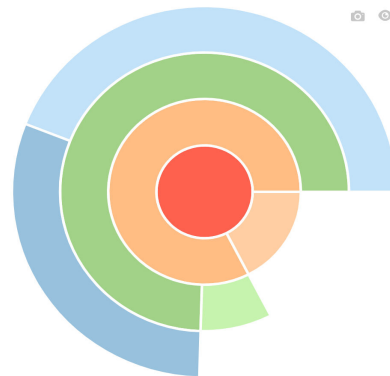
Valid PID Reads

**175,086,793**

Unique PID Reads

Key Metrics

|                       |             |       |
|-----------------------|-------------|-------|
| Total Reads           | 573,659,235 | 100%  |
| Valid CID Reads       | 473,613,579 | 82.6% |
| Valid PID Reads       | 427,761,398 | 90.3% |
| Unique PID Reads      | 175,086,793 | 40.9% |
| Sequencing Saturation | 252,674,605 | 59.1% |
| Invalid PID Reads     | 45,852,181  | 9.7%  |
| Invalid CID Reads     | 100,045,656 | 17.4% |



Information

|         |                     |        |        |
|---------|---------------------|--------|--------|
| Species | mouse               | Tissue | thymus |
| FASTQ   | E150023160_L01_11_1 |        |        |



### I Sequencing ?

|                                  |             |
|----------------------------------|-------------|
| Total Reads                      | 573,659,235 |
| Rate of Q30 Bases in CID         | 87.72%      |
| Rate of Q30 Bases in MID         | 88.1%       |
| Rate of Q30 Bases in PID         | 93.44%      |
| Rate of Valid PID Reads in Total | 74.57%      |

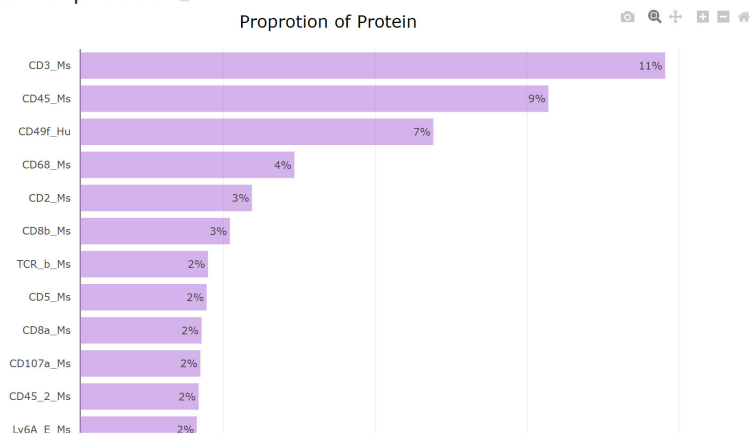
### I Protein Mapping ?

|                           |             |
|---------------------------|-------------|
| Total Unique PID Reads    | 175,086,793 |
| Ratio of Unique PID Reads | 40.9%       |

### I Tissue ?

|                                      |             |
|--------------------------------------|-------------|
| DNB Under Tissue                     | 48,203,139  |
| Protein-Captured DNBs Under Tissue   | 24,749,871  |
| Number of MID Under Tissue Coverage  | 113,480,216 |
| Fraction MID in Spots Under Tissue   | 64.84%      |
| Reads Under Tissue                   | 310,743,393 |
| Fraction Reads in Spots Under Tissue | 65.41%      |

### I Protein Proportion ?



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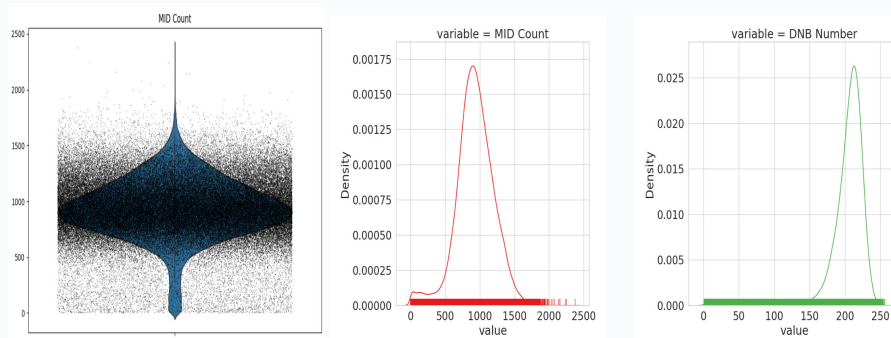
Alerts

- Summary
  - Gene
  - Protein
- Square Bin
  - Gene
  - Protein
- Cell Bin
  - Gene
  - Protein
- Image
- Analysis

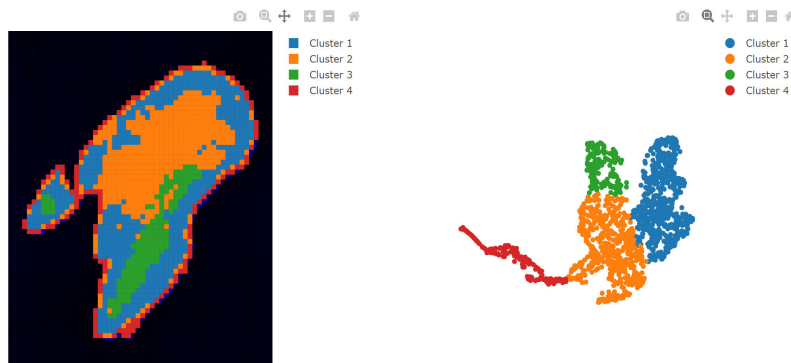
Tissue Square Bin Statistics

| Bin Size | Mean Reads (per bin) | Median Reads (per bin) | Mean MID (per bin) | Median MID (per bin) |
|----------|----------------------|------------------------|--------------------|----------------------|
| 1        | 12.42                | 10                     | 4.59               | 4                    |
| 20       | 2,551                | 2,541                  | 931                | 930                  |
| 50       | 15,683               | 15,822                 | 5,727              | 5,791                |
| 100      | 61,133               | 62,505                 | 22,325             | 22,867               |
| 150      | 133,883              | 139,390                | 48,892             | 51,058               |
| 200      | 233,115              | 245,086                | 85,131             | 89,608               |

Bin 20



Clustering



show image — show cluster

Tissue Plot with Spots (bin200)

UMAP Projection of Spots (bin200)

时空组学 STOmics Analytical Report

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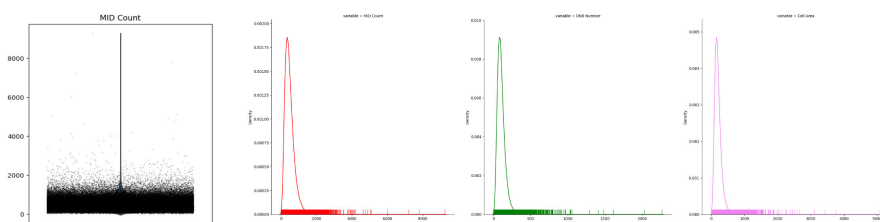
I Alerts

- Summary
  - Gene
  - Protein
- Square Bin
  - Gene
  - Protein
- Cell Bin**
  - Gene
  - Protein**
- Image
- Analysis

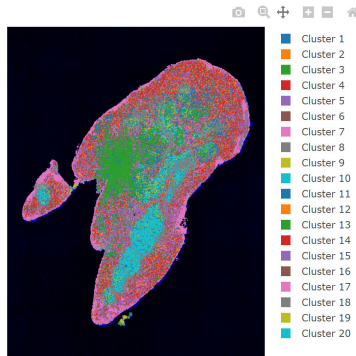
I Cell Bin Statistics

|                |         |                  |     |
|----------------|---------|------------------|-----|
| Cell Count     | 198,568 |                  |     |
| Mean Cell Area | 203     | Median Cell Area | 183 |
| Mean DNB Count | 104     | Median DNB Count | 95  |
| Mean MID       | 482     | Median MID       | 430 |

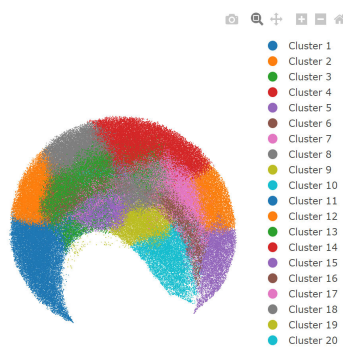
I Cell Bin



I Clustering



Tissue Plot with Spots (cell bin)



UMAP Projection of Spots (cellbin)





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Alerts

- Summary
  - Gene
  - Protein
- Square Bin
  - Gene
  - Protein
- Cell Bin
  - Gene
  - Protein
- Image**
- Analysis

Image Information

|                    |                                   |
|--------------------|-----------------------------------|
| Manufacture        | motic                             |
| Model              | MoticamProS5 Lite M               |
| Scan Time          | 2023-7-13 16:14:11                |
| Overlap            | 0.1                               |
| Exposure Time (ms) | 116.513                           |
| Scan Rows          | 9                                 |
| Scan Columns       | 7                                 |
| FOV Height         | 2,026                             |
| FOV Width          | 2,420                             |
| Stain Type         | DAPI                              |
| Stitched Image     | No                                |
| Image Name         | A02677B5_SC_20240131_192213_3.0.3 |

QC

|                           |       |
|---------------------------|-------|
| Image QC Version          | 3.0.3 |
| QC Pass                   | Pass  |
| Track Line Score          | 98    |
| Clarity Score             | 21    |
| Good FOV Count            | 55    |
| Total FOV Count           | 63    |
| Stitching Score           | 76    |
| Tissue Segmentation Score | 20.0  |
| Registration Score        | 91    |

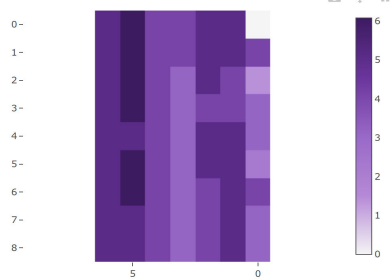
Stitching

|                            |        |
|----------------------------|--------|
| Template Source Row No.    | 1      |
| Template Source Column No. | 0      |
| Global Height              | 16,618 |
| Global Width               | 15,488 |

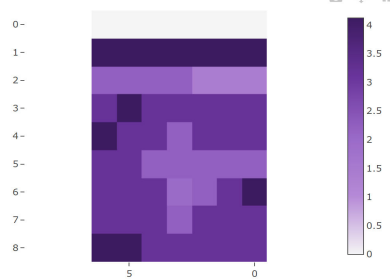
Registration

|                            |        |
|----------------------------|--------|
| ScaleX                     | 0.989  |
| ScaleY                     | 0.988  |
| Rotation                   | -1.634 |
| Flip                       | True   |
| Image X Offset             | 5,349  |
| Image Y Offset             | 5,936  |
| Manual ScaleX              | -      |
| Manual ScaleY              | -      |
| Manual Rotation            | -      |
| Counter Clockwise Rotation | 0      |
| Matrix X Offset            | 0.00   |
| Matrix Y Offset            | 0.00   |
| Matrix Height              | 26,460 |
| Matrix Width               | 26,460 |

Horizontal Image Stitching Deviation Heatmap



Vertical Image Stitching Deviation Heatmap

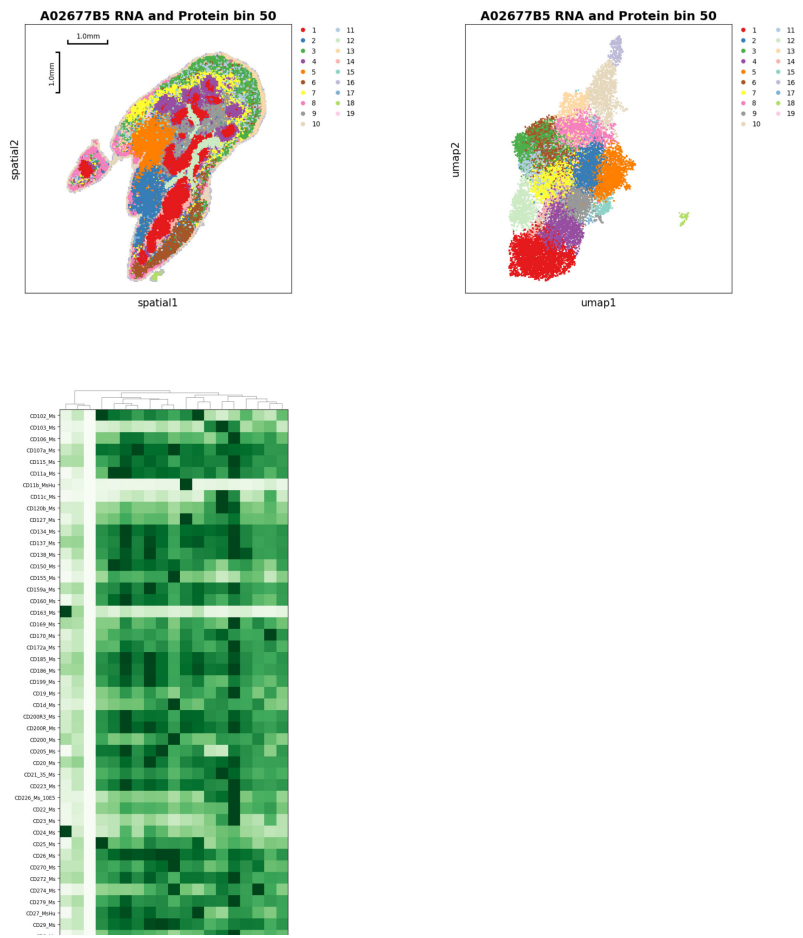


A02677B5

Alerts

- Summary
  - Gene
  - Protein
- Square Bin
  - Gene
  - Protein
- Cell Bin
  - Gene
  - Protein
- Image
- Analysis

Multomics Clustering and Heatmap





## Appendices

### Appendix A: Recommended Directory Structure for Raw Data



Recommend to organize raw data as below if users prefer to use **SAW shell script** provided on the **SAW GitHub page** (<https://github.com/STOmics/SAW>).

#### Spatial Transcriptomics (ST) :

```
$ tree
.
|-- image
|   |-- SS200000135TL_D1_SC_20230822_144400_3.0.0.ipr
|   `-- SS200000135TL_D1_SC_20230822_144400_3.0.0.tar.gz
|-- mask
|   `-- SS200000135TL_D1.barcodeToPos.h5
|-- md5
|-- reads
|   |-- E100026571_L01_trim_read_1.fq.gz
|   `-- E100026571_L01_trim_read_2.fq.gz
`-- reference
    |-- STAR_SJ100
    |   |-- chrLength.txt
    |   |-- chrNameLength.txt
    |   |-- chrName.txt
    |   |-- chrStart.txt
    |   |-- exonGeTrInfo.tab
    |   |-- exonInfo.tab
    |   |-- FIndex
    |   |-- geneInfo.tab
    |   |-- Genome
    |   |-- genomeParameters.txt
    |   |-- SA
    |   |-- SAindex
    |   |-- SAindexAux
    |   |-- sjdbInfo.txt
    |   |-- sjdbList.fromGTF.out.tab
    |   |-- sjdbList.out.tab
    |   `-- transcriptInfo.tab
    |-- genes.gtf
    `-- genome.fa

5 directories, 25 files
```

## Spatial Proteomics &amp; Transcriptomics (PT) :

```
$ tree
.
|-- image
|   |-- A02677B5_SC_20240131_192213_3.0.3.ipr
|   `-- A02677B5_SC_20240131_192213_3.0.3.tar.gz
|-- mask
|   `-- A02677B5.barcodeToPos.h5
|-- md5
|-- STOmics-RNA
|   |-- V350248064_L01_read_1.fq.gz
|   |-- V350248064_L01_read_2.fq.gz
|   |-- V350248064_L02_read_1.fq.gz
|   |-- V350248064_L02_read_2.fq.gz
|   |-- V350248064_L03_read_1.fq.gz
|   `-- V350248064_L03_read_2.fq.gz
|-- STOmics-ADT
|   |-- E150023160_L01_11_1.fq.gz
|   `-- E150023160_L01_11_2.fq.gz
|-- protein-reference
|   `-- ProteinPanel_128_mouse.list
`-- reference
    |-- STAR_SJ100
    |   |-- chrLength.txt
    |   |-- chrNameLength.txt
    |   |-- chrName.txt
    |   |-- chrStart.txt
    |   |-- exonGeTrInfo.tab
    |   |-- exonInfo.tab
    |   |-- FMindex
    |   |-- geneInfo.tab
    |   |-- Genome
    |   |-- genomeParameters.txt
    |   |-- SA
    |   |-- SAindex
    |   |-- SAindexAux
    |   |-- sjdbInfo.txt
    |   |-- sjdbList.fromGTF.out.tab
    |   |-- sjdbList.out.tab
    |   `-- transcriptInfo.tab
    |-- genes.gtf
    `-- genome.fa
```

7 directories, 32 files

## Appendix B: SAW ST Output File List

| STEP                   | OUTPUT  | FILE DESCRIPTION  |
|------------------------|---|---|
| <b>splitMask</b>       | *.SN.barcodeToPos.bin   | Split Stereo-seq Chip T mask file according to the CID.   |
| <b>mapping</b>         | lane.Aligned.sortedByCoord.out.bam  | Binary Alignment/Map file, used for storing the sequence alignment information.   |
|                        | lane.barcodeReadsCount.txt  | Mapped CID list file with reads counts for each CID, the three columns record x, y, and read count.   |
|                        | lane.Log.final.out  | Summary mapping statistics after mapping job is complete (STAR output)  |
|                        | lane.Log.out  | Main log file in STAR mapping (STAR output)   |
|                        | lane.Log.progress.out   | Reports job process statistics (STAR output)  |
|                        | lane.SJ.out.tab   | Splice junctions detected in the mapping (STAR output)  |
|                        | lane.bcPara   | A parameters file defines CID mapping options   |
|                        | lane.CIDMap.stat  | Statistical report of CID mapping, such as CID mapped reads count, reads sequencing quality, mapped DNB count, etc.   |
|                        | lane.run.log  | Log file of mapping module.   |
|                        | lane.valid_cid_reads.fq   | <b>Valid CID Reads</b> in FASTQ format after CID mapping. Similar to SE FASTQ format but with different first row of each read, for instance, "@V350044321L1C001R0020993658 Cx:i:10413 Cy:i:7737 D3450E0D391E EC7FF", where Cx and Cy represent the decoded coordinates and are added after readID.   |
| lane.unmapped_reads.fq | Un-mapped reads in FASTQ format after mapping <b>Clean Reads</b> to the reference genome. Similar to SE FASTQ format but with different first row of each read, for instance, "@V350044321L1C001R0020993658 Cx:i:10413 Cy:i:7737 D3450E0D391E EC7FF", where Cx and Cy represent the decoded coordinates and are added after readID. |   |
| <b>merge</b>           | SN.merge.barcodeReadsCount.txt  | Mapped CID list file with reads counts for each CID., the three columns record x, y, and read count.  |
| <b>count</b>           | SN.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam   | Annotated BAM file sorted by coordinate, includes uniquely mapped reads and multi-mapped reads whose HI:i tag is 1.   |
|                        | SN.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam.csi   | Index file of annotated BAM.  |
|                        | SN.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam.summary.stat  | Statistical summary report file for count. Total reads in filter&deduplication metrics stand for the total alignment record count in BAM. Pass filter and total reads in annotation metrics are the same, they represent uniquely mapped reads that will be used for annotation, MID correction and quantification.   |
|                        | SN.raw.gef  | Gene expression file in hdf5 format. This is the first raw matrix that includes the expression information over a complete chip region. It only includes geneExp group for the bin size of 1. The origin of expression matrix has been calibrated to (0,0), and the offset x and y has been record as minX and minY in the attribute of geneExp/expression dataset. |
|                        | SN_raw_barcode_gene_exp.txt   | A space-separate file records coordinate, gene, MID, and count information. Which is prepared to be a sampling file that performs sequence saturation. The 5 columns are y, x, geneIndex, MIDIndex, readCount.  |
|                        | count_data_hhmmss.log   | Log file.   |

| STEP                         | OUTPUT  | FILE DESCRIPTION  |
|------------------------------|---|---|
|                              | date-hh-mm-ss.log   | Log file.   |
|                              | SN or other user specified name for the image folder used when input into ImageStudio | This subdirectory stores raw small image pieces in TIFF format.   |
|                              | SN_0000_0000_YYYY-MM-DD_hh-mm-ss-n.tif  | Small image pieces in TIFF format.  |
|                              | <stainType>_fov_stitched_transformed.rpi  | Multiple staining stitched panoramic images (.tif) are stored in RPI file. The stitched panoramic image that has pre-registered with the track line pattern template. So that it will not need to further rotate a non-right angle or scale.  |
|                              | fov_stitched.rpi  | Multiple staining stitched panoramic images (.tif) are stored in RPI file. The stitched panoramic image. It needs to further rotate a non-right angle or scale.   |
|                              | <stainType>_fov_stitched_transformed.tif  | The stitched panoramic image from DAPI/IF layers that has pre-registered with the track line pattern template. So that it will not need to further rotate a non-right angle or scale.   |
| register& imageTools ipr2img | <stainType>_fov_stitched.tif  | The stitched panoramic image. It needs to further rotate a non-right angle or scale.  |
|                              | <stainType>matrix_template.txt  | Track line cross point template for the registered staining image. Used for assessing registration result.  |
|                              | SN_<chipType>date_time_version.ipr  | IPR format image process record file which records basic image information gathered from ImageStudio.   |
|                              | <stainType>_SN_mask.tif   | Registered cell segmentation binary image file from staining layers in TIFF format.   |
|                              | <stainType>_SN_regist.tif   | Registered panoramic image file from staining layers in TIFF format.  |
|                              | SN.rpi  | Registered panoramic image file, tissue area boundary, and cell boundaries (downsampled) that stores as an image pyramid.   |
|                              | <stainType>_SN_tissue_cut.tif   | The tissue segmentation result of registered panoramic image file from staining layers in TIFF format.  |
|                              | <stainType>_transform_template.txt  | Track line cross point template for the <stainType>_fov_stitched_transformed.tif. Used for assessing stitching result.  |
| tissueCut                    | SN.gef  | A gene expression file in hdf5 format. This file is a complete GEF format which includes geneExp group and wholeExp group in bin1, 10, 20, 50, 100, 200, and 500. It also includes a stat group. The original expression matrix has been calibrated to (0,0), and the offset x and y has been recorded as minX and minY in the attribute of geneExp/expression dataset. |
|                              | SN.tissue.gef   | A gene expression file in hdf5 format. Tissue GEF includes the expression information of the tissue coverage region. It only includes geneExp group for the bin size of 1. The origin of expression matrix has been calibrated to (0,0), and the offset x and y has been record as minX and minY in the attribute of geneExp/expression dataset same to raw GEF.        |

| STEP             | OUTPUT   | FILE DESCRIPTION  |
|------------------|--|---|
|                  | <code>SN.&lt;label&gt;.raw.label.gef</code>      | A gene expression file in hdf5 format. It carries the expression information of the labeled tissue coverage region, with only geneExp group for the bin size of 1. The origin of expression matrix has been calibrated to (0,0), and the offset x and y has been recorded as minX and minY in the attribute of geneExp/expression dataset same to raw GEF.  |
|                  | <code>SN.&lt;label&gt;.label.gef</code>          | A gene expression file in hdf5 format. It includes the expression information of the labeled tissue coverage region, with geneExp group and wholeExp group in bin1, 10, 20, 50, 100, 200, and 500. It also includes a stat group. The original expression matrix has been calibrated to (0,0), and the offset x and y have been recorded as minX and minY in the attribute of geneExp/expression dataset. |
|                  | <code>tissue_fig</code>                          | This directory stores the statistical plots for the tissue coverage region  |
|                  | <code>scatter_100x100_MID_gene_counts.png</code> | Scatter plot of MID count and gene types in each bin (bin100)   |
|                  | <code>scatter_150x150_MID_gene_counts.png</code> | Scatter plot of MID count and gene types in each bin (bin150)   |
|                  | <code>scatter_200x200_MID_gene_counts.png</code> | Scatter plot of MID count and gene types in each bin (bin200)   |
|                  | <code>scatter_20x20_MID_gene_counts.png</code>   | Scatter plot of MID count and gene types in each bin (bin20)  |
|                  | <code>scatter_50x50_MID_gene_counts.png</code>   | Scatter plot of MID count and gene types in each bin (bin50)  |
|                  | <code>statistic_100x100_MID_gene_DNB.png</code>  | Univariate distribution of MID count, gene types, and DNB numbers with rug along the x axis (bin100)  |
| <b>tissueCut</b> | <code>statistic_150x150_MID_gene_DNB.png</code>  | Univariate distribution of MID count, gene types, and DNB numbers with rug along the x axis (bin150)  |
|                  | <code>statistic_200x200_MID_gene_DNB.png</code>  | Univariate distribution of MID count, gene types, and DNB numbers with rug along the x axis (bin200)  |
|                  | <code>statistic_20x20_MID_gene_DNB.png</code>    | Univariate distribution of MID count, gene types, and DNB numbers with rug along the x axis (bin20)   |
|                  | <code>statistic_50x50_MID_gene_DNB.png</code>    | Univariate distribution of MID count, gene types, and DNB numbers with rug along the x axis (bin50)   |
|                  | <code>violin_100x100_MID_gene.png</code>         | Violin plots show the distribution of deduplicated MID count and gene types in each bin (bin100)  |
|                  | <code>violin_150x150_MID_gene.png</code>         | Violin plots show the distribution of deduplicated MID count and gene types in each bin (bin150)  |
|                  | <code>violin_200x200_MID_gene.png</code>         | Violin plots show the distribution of deduplicated MID count and gene types in each bin (bin200)  |
|                  | <code>violin_20x20_MID_gene.png</code>           | Violin plots show the distribution of deduplicated MID count and gene types in each bin (bin20)   |



| STEP                   | OUTPUT  | FILE DESCRIPTION  |
|------------------------|---|---|
|                        | <code>violin_50x50_MID_gene.png</code>                | Violin plots show the distribution of deduplicated MID count and gene types in each bin (bin50)   |
|                        | <code>tissuecut.stat</code>                           | Statistical report for tissue coverage region.  |
|                        | <code>&lt;label&gt;.tissuecut.stat</code>             | Statistical report for labeling coverage region.  |
| <b>cellCut</b>         | <code>SN.cellbin.gef</code>                           | A gene expression file for cells in hdf5 format. Cellbin GEF includes the expression information of the cells, such as the coordinate of the centroid, boundary coordinates, expression of genes, and cell area. The cell is recorded by its boundary. The origin of expression matrix has been calibrated to (0,0), and the coordinate offset x and y have been recorded as offsetX and offsetY in the attribute of the GEF file, the same as minX and minY in the raw GEF file. |
|                        | <code>SN.adjusted.cellbin.gef</code>                  | Cellbin GEF generated from adjusted cell segmentation TIFF image and *.raw.gef.   |
| <b>cellCorrect</b>     | <code>SN.adjusted.gem</code>                          | Cellbin GEM generated from adjusted cell segmentation TIFF image and *.raw.gef.   |
|                        | <code>&lt;stainType&gt;_SN_mask_edm_dis_10.tif</code> | Adjusted cell segmentation binary image in TIFF format.   |
| <b>spatial-Cluster</b> | <code>SN.bin200_1.0.spatial.cluster.h5ad</code>       | H5AD file for spatial clustering analysis result.   |
|                        | <code>SN.cell.cluster.h5ad</code>                     | H5AD file for cell clustering analysis result.  |
| <b>cellCluster</b>     | <code>SN.adjusted.cell.cluster.h5ad</code>            | H5AD file for cell clustering analysis result, based on adjusted Cellbin GEF.   |
|                        | <code>plot_1x1_saturation.png</code>                  | Sequencing saturation analysis plots for bin1. Calculated by 1-(unique reads/total reads) for each bin (bin1).  |
|                        | <code>plot_200x200_saturation.png</code>              | Sequencing saturation analysis plots for bin200. Calculated by 1-(unique reads/total reads) for each bin (bin1).  |
| <b>saturation</b>      | <code>sequence_saturation.tsv</code>                  | Sequence saturation file. The nine columns are sampling component (#sample), bin1 total reads (bar_x), sequence saturation value at bin1 (bar_y1), median gene count at bin1 (bar_y2), unique reads at bin1 (bar_umi), bin200 total reads (bin_x), sequence saturation value at bin200 (bin_y1), median gene count at bin200 (bin_y2), and unique reads at bin200 (bin_umi), respectively.  |
|                        | <code>SN.report.html</code>                           | HTML analytical report.   |
|                        | <code>SN.statistics.json</code>                       | Statistical summary report in JSON format. It gathers all important statistical metrics from the statistical report in each step.   |
| <b>report</b>          | <code>scatter_1x1_MID_gene_counts.png</code>          | Scatter plot of MID count and gene types in each cell.  |
|                        | <code>statistic_1x1_cell_area.png</code>              | Univariate distribution of cell area with rug along the x axis of each cell.  |

| STEP | OUTPUT                        | FILE DESCRIPTION   |
|------|-------------------------------|--|
|      | <b>statistic_1x1_DNB.png</b>  | Univariate distribution of DNB numbers with rug along the x axis of each cell. |
|      | <b>statistic_1x1_gene.png</b> | Univariate distribution of gene types with rug along the x axis of each cell.  |
|      | <b>statistic_1x1_MID.png</b>  | Univariate distribution of MID count with rug along the x axis of each cell.   |
|      | <b>violin_1x1_gene.png</b>    | Violin plots show the distribution of gene types in each cell.                 |
|      | <b>violin_1x1_MID.png</b>     | Violin plots show the distribution of deduplicated MID count in each cell.     |

## Appendix C: SAW PT Output File List

| STEP                   | OUTPUT  | FILE DESCRIPTION  |
|------------------------|---|---|
| <b>splitMask</b>       | *.SN.barcodeToPos.bin   | Split Stereo-seq Chip T mask file according to the CID.   |
| <b>mapping</b>         | lane.Aligned.sortedByCoord.out.bam  | Binary Alignment/Map file, used for storing the sequence alignment information.   |
|                        | lane.barcodeReadsCount.txt  | Mapped CID list file with reads counts for each CID, the three columns record x, y, and read count.   |
|                        | lane.Log.final.out  | Summary mapping statistics after mapping job is complete (STAR output)  |
|                        | lane.Log.out  | Main log file in STAR mapping (STAR output)   |
|                        | lane.Log.progress.out   | Reports job process statistics (STAR output)  |
|                        | lane.SJ.out.tab   | Splice junctions detected in the mapping (STAR output)  |
|                        | lane.bcPara   | A parameters file defines CID mapping options   |
|                        | lane.CIDMap.stat  | Statistical report of CID mapping, such as CID mapped reads count, reads sequencing quality, mapped DNB count, etc.   |
|                        | lane.run.log  | Log file of mapping module.   |
|                        | lane.valid_cid_reads.fq   | <b>Valid CID Reads</b> in FASTQ format after CID mapping. Similar to SE FASTQ format but with different first row of each read, for instance, "@V350044321L1C001R0020993658 Cx:i:10413 Cy:i:7737 D3450E0D391E EC7FF", where Cx and Cy represent the decoded coordinates and are added after readID.   |
| lane.unmapped_reads.fq | Un-mapped reads in FASTQ format after mapping <b>Clean Reads</b> to the reference genome. Similar to SE FASTQ format but with different first row of each read, for instance, "@V350044321L1C001R0020993658 Cx:i:10413 Cy:i:7737 D3450E0D391E EC7FF", where Cx and Cy represent the decoded coordinates and are added after readID. |   |
| <b>merge</b>           | SN.merge.barcodeReadsCount.txt  | Mapped CID list file with reads counts for each CID., the three columns record x, y, and read count.  |
| <b>count</b>           | SN.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam   | Annotated BAM file sorted by coordinate, includes uniquely mapped reads and multi-mapped reads whose HI:i tag is 1.   |
|                        | SN.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam.csi   | Index file of annotated BAM.  |
|                        | SN.Aligned.sortedByCoord.out.merge.q10.dedup.target.bam.summary.stat  | Statistical summary report file for count. Total reads in filter&deduplication metrics stand for the total alignment record count in BAM. Pass filter and total reads in annotation metrics are the same, they represent uniquely mapped reads that will be used for annotation, MID correction and quantification.   |
|                        | SN.raw.gef  | Gene expression file in hdf5 format. This is the first raw matrix that includes the expression information over a complete chip region. It only includes geneExp group for the bin size of 1. The origin of expression matrix has been calibrated to (0,0), and the offset x and y has been record as minX and minY in the attribute of geneExp/expression dataset. |
|                        | SN_raw_barcode_gene_exp.txt   | A space-separate file records coordinate, gene, MID, and count information. Which is prepared to be a sampling file that performs sequence saturation. The 5 columns are y, x, geneIndex, MIDIndex, readCount.  |
|                        | count_data_hhmmss.log   | Log file.   |

| STEP                                    | OUTPUT  | FILE DESCRIPTION  |
|---|---|---|
|   | date-hh-mm-ss.log   | Log file.   |
|   | SN or other user specified name for the image folder used when input into ImageStudio | This subdirectory stores raw small image pieces in TIFF format.   |
|   | SN_0000_0000_YYYY-MM-DD_hh-mm-ss-n.tif  | Small image pieces in TIFF format.  |
|   | <stainType>_fov_stitched_transformed.rpi  | Multiple staining stitched panoramic images (.tif) are stored in RPI file. The stitched panoramic image that has pre-registered with the track line pattern template. So that it will not need to further rotate a non-right angle or scale.  |
|   | fov_stitched.rpi  | Multiple staining stitched panoramic images (.tif) are stored in RPI file. The stitched panoramic image. It needs to further rotate a non-right angle or scale.   |
|   | <stainType>_fov_stitched_transformed.tif  | The stitched panoramic image from DAPI/IF layers that has pre-registered with the track line pattern template. So that it will not need to further rotate a non-right angle or scale.   |
| <b>register&amp; imageTools ipr2img</b> | <stainType>_fov_stitched.tif  | The stitched panoramic image. It needs to further rotate a non-right angle or scale.  |
|   | <stainType>matrix_template.txt  | Track line cross point template for the registered staining image. Used for assessing registration result.  |
|   | SN_<chipType>date_time_version.ipr  | IPR format image process record file which records basic image information gathered from ImageStudio.   |
|   | <stainType>_SN_mask.tif   | Registered cell segmentation binary image file from staining layers in TIFF format.   |
|   | <stainType>_SN_regist.tif   | Registered panoramic image file from staining layers in TIFF format.  |
|   | SN.rpi  | Registered panoramic image file, tissue area boundary, and cell boundaries (downsampled) that stores as an image pyramid.   |
|   | <stainType>_SN_tissue_cut.tif   | The tissue segmentation result of registered panoramic image file from staining layers in TIFF format.  |
|   | <stainType>_transform_template.txt  | Track line cross point template for the <stainType>_fov_stitched_transformed.tif. Used for assessing stitching result.  |
| <b>calibration</b>                      | SN.gef/<br>SN.protein.gef   | A gene expression file in hdf5 format. This file is a complete GEF format which includes geneExp group and wholeExp group in bin1, 10, 20, 50, 100, 200, and 500. It also includes a stat group. The offset x and y have been recorded as minX and minY in the attribute of geneExp/expression dataset. |
|   | SN.calibrated.raw.gef/<br>SN.protein.calibrated.raw.gef                               | Gene/Protein expression file in hdf5 format, that includes the expression information over a complete chip region. It only includes geneExp group for the bin size of 1. These two expression matrices have been calibrated to the same offset.   |

| STEP             | OUTPUT  | FILE DESCRIPTION  |
|------------------|---|---|
|                  | <code>SN.&lt;label&gt;.raw.label.gef/<br/>SN.protein.&lt;label&gt;.raw.<br/>label.gef</code>  | A gene/protein expression file in hdf5 format. It carries the expression information of the labeled tissue coverage region, with only gene-Exp group for the bin size of 1. The origin of expression matrix has been calibrated to (0,0), and the offset x and y has been recorded as minX and minY in the attribute of geneExp/expression dataset same to raw GEF. |
|                  | <code>SN.&lt;label&gt;.label.gef/<br/>SN.protein.&lt;label&gt;.<br/>label.gef</code>  | A gene/protein expression file in hdf5 format. It carries the expression information of the labeled tissue coverage region, with only geneExp group for the bin size of 1. The origin of expression matrix has been calibrated to (0,0), and the offset x and y has been recorded as minX and minY in the attribute of geneExp/expression dataset same to raw GEF.  |
|                  | <code>tissue_fig</code>   | This directory stores the statistical plots for the tissue coverage region  |
|                  | <code>scatter_&lt;bin&gt;x&lt;bin&gt;<br/>_MID_gene_counts.png</code>   | Scatter plot of MID count and gene types in each bin (bin200/bin150/bin100/bin50/bin20).  |
| <b>tissueCut</b> | <code>statistic_&lt;bin&gt;x&lt;bin&gt;<br/>_MID_gene_DNB.png /<br/>statistic_&lt;bin&gt;x&lt;bin&gt;<br/>_MID.png /<br/>statistic_&lt;bin&gt;x&lt;bin&gt;<br/>_gene.png /<br/>statistic_&lt;bin&gt;x&lt;bin&gt;<br/>_MID_gene_DNB.png</code> | Univariate distribution of MID count, gene types, and DNB numbers with rug along the x axis (bin200/bin150/bin100/bin50/bin20).   |
|                  | <code>violin_&lt;bin&gt;x&lt;bin&gt;<br/>_MID_gene.png /<br/>violin_&lt;bin&gt;x&lt;bin&gt;<br/>_MID.png /<br/>violin_&lt;bin&gt;x&lt;bin&gt;<br/>_gene.png</code>  | Violin plots show the distribution of deduplicated MID count and gene types in each bin (bin200/bin150/bin100/bin50/bin20).   |
|                  | <code>tissuecut.stat</code>   | Statistical report for tissue coverage region.  |
|                  | <code>&lt;label&gt;.tissuecut.stat</code>   | Statistical report for labeling coverage region.  |
|                  | <code>SN.tissue.gef/<br/>SN.protein.tissue.gef</code>   | A gene/protein expression file in hdf5 format. It carries the expression information of the labeled tissue coverage region, with only geneExp group for the bin size of 1. The origin of expression matrix has been calibrated to (0,0), and the offset x and y has been recorded as minX and minY in the attribute of geneExp/expression dataset same to raw GEF.  |

| STEP  | OUTPUT   | FILE DESCRIPTION  |
|---|--|---|
| <b>cellCut</b>  | SN.cellbin.gef /<br>SN.protein.cellbin.gef   | A gene expression file for cells in hdf5 format. Cellbin GEF includes the expression information of the cells, such as the coordinate of the centroid, boundary coordinates, expression of genes, and cell area. The cell is recorded by its boundary. The origin of expression matrix has been calibrated to (0,0), and the coordinate offset x and y have been recorded as offsetX and offsetY in the attribute of the GEF file, the same as minX and minY in the raw GEF file. |
| <b>cellCorrect</b>                                      | SN.adjusted.cellbin<br>.gef / SN.protein.<br>adjusted.cellbin.gef                          | Cellbin GEF generated from adjusted cell segmentation TIFF image and *.raw.gef.   |
|   | SN.adjusted.gem /<br>SN.protein.adjusted.gem   | Cellbin GEM generated from adjusted cell segmentation TIFF image and *.raw.gef.   |
|   | <stainType>_SN_mask_<br>edm_dis_10.tif   | Adjusted cell segmentation binary image in TIFF format.   |
| <b>spatial-Cluster<br/>&amp;<br/>spatial-Cluster-SP</b> | SN.bin200_1.0.spatial.<br>cluster.h5ad /<br>SN_bin200_0.1.protein.<br>spatial.cluster.h5ad | H5AD file for spatial clustering analysis result.   |
| <b>cellCluster<br/>&amp;<br/>cellCluster-SP</b>         | SN.cell.cluster.h5ad /<br>SN.protein.cell.<br>cluster.h5ad                                 | H5AD file for cell clustering analysis result.  |
|   | SN.adjusted.cell.<br>cluster.h5ad /<br>SN.protein.adjusted.<br>cell.cluster.h5ad           | H5AD file for cell clustering analysis result, based on adjusted Cellbin GEF.   |
| <b>saturation</b>                                       | plot_1x1_saturation.<br>png  | Sequencing saturation analysis plots for bin1. Calculated by 1-(unique reads/total reads) for each bin (bin1).  |
|   | plot_200x200_satura-<br>tion.png   | Sequencing saturation analysis plots for bin200. Calculated by 1-(unique reads/total reads) for each bin (bin1).  |
|   | sequence_saturation.<br>tsv  | Sequence saturation file. The nine columns are sampling component (#sample), bin1 total reads (bar_x), sequence saturation value at bin1 (bar_y1), median gene count at bin1 (bar_y2), unique reads at bin1 (bar_umi), bin200 total reads (bin_x), sequence saturation value at bin200 (bin_y1), median gene count at bin200 (bin_y2), and unique reads at bin200 (bin_umi), respectively.  |

| STEP                | OUTPUT                                   | FILE DESCRIPTION   |
|---------------------|--|--|
| multiomics-Analysis | SN_50_differential_expression.csv        | The CSV file records the 1-vs-all differential expression analysis results, which includes proteins and RNA.   |
|                     | SN_50_dotplot_RNA_totalVI_03.png         | Heatmap of RNA within the latent space clusters.   |
|                     | SN_50_matrixplot_Protein_totalVI_04.png  | Heatmap of proteins within the latent space clusters.  |
|                     | SN_50_Protein_Correlation_Heatmap_05.png | Correlation plot of protein-vs-protein.  |
|                     | SN_50_spatial_leiden_totalVI_02.png      | Visualized spatial cluster plot of latent space.   |
|                     | SN_50_UMAP_leiden_totalVI_01.png         | UMAP plot of latent space.   |
|                     | SN_50.h5mu                               | H5MU file records proteins and RNA clustering analysis results.  |
| report-PT           | rna_cell                                 | The directory stores the cellbin statistical plots under tissue (Transcriptomics)  |
|                     | protein_cell                             | The directory stores the cellbin statistical plots under tissue (Proteomics)   |
|                     | AnalysisReport                           | The directory stores all the elements of the workflow summary report. The AnalysisReport/report.html is the homepage.  |
|                     | input.yaml                               | The configure file records all input files.  |
|                     | SN.statistics.json                       | Statistical summary report in JSON format. It gathers all important statistical metrics from the statistical report in each step.  |
| mapping-SP          | SN_cid_pid_mid_reads.tsv                 | A tab-separate file records coordinate, gene, MID, PIDIndex and count information. Which is prepared to be a sampling file that performs sequence saturation for all proteins. The 5 columns are x, y, PIDIndex, MIDIndex, readCount.  |
|                     | SN_map.stat                              | Statistical report of CID mapping and filtration, MID filtration, PID mapping and sequencing quality.  |
|                     | SN.protein.gem.gz                        | Protein expression file in GEM format.   |
|                     | SN.protein.raw.gef                       | Protein expression file in hdf5 format. This is the first raw matrix that includes the expression information over a complete chip region. It only includes the geneExp group for the bin size of 1. The origin of expression matrix has been calibrated to (0,0), and the offset x and y has been record as minX and minY in the attribute of geneExp/expression dataset. |
|                     | SN_valid_cid_reads.tsv                   | Mapped CID list file with reads counts for each CID, the three columns record x, y, and read count.  |

## Appendix D: Handling Errors and Exceptions

### Common Errors

- **File access error:**  
Some examples of file access error:

```
terminate called after throwing an instance of 'std::invalid_argument'  
what(): Could not open the file: xxxxxx  
...
```

Or

```
#006: H5FDsec2.c line 352 in H5FD__sec2_open(): unable to open file: name = '/path/  
to/hdf5/file', errno = 2, error message = 'No such file or directory', flags = 0,  
o_flags = 0  
major: File accessibility  
minor: Unable to open file  
...
```



Solution:

```
# Attempt 1: Bind file path before execute command.  
$ export SINGULARITY_BIND="/path/to/file/directory"  
  
# Attempt 2: Turn off the HDF5 lock on the file by running the command below before  
running SAW.  
$ export HDF5_USE_FILE_LOCKING=FALSE
```



## mapping

- **readNameSeparator error:**

```
EXITING: FATAL INPUT ERROR: empty value for parameter "readNameSeparator" in input
"Command-Line"
SOLUTION: use non-empty value for this parameter
```

 try to delete `--readNameSeparator \` from the command.


- **limitBAMsortRAM error:**

```
Error code: SAW-A10183
EXITING because of fatal ERROR: not enough memory for BAM sorting:
SOLUTION: re-run STAR with at least -limitBAMsortRAM xxxxxxxxx
```

 Modify the value specified by the `--limitBAMsortRAM` refer to the SOLUTION in the stderr.

- **limitOutSJcollapsed error:**


```
Error code: SAW-A10182
EXITING because of fatal error: buffer size of SJ output is too small
Solution: increase input parameter --limitOutSJcollapsed
```

 the parameters `--limitOutSJcollapsed` and `--limitIObufferSize` are paired. Please replace them with `--limitOutSJcollapsed 10000000 --limitIObufferSize=480000000` and try again.

## imageTools

- **OSError raises for imageTools merge**

```
OSError: [Errno 30] Read-only file system: '/opt/saw_v5.4.0_software/pipeline/im-
ageTools/imagetools-1.0.0/log'
```

 Error raises because the `-o` input is a filename without path. Try to input `/path/to/output/file.rpi` or `./file.rpi` for `-o`.

## Appendix E: Error Codes

SAW error codes are designed to catch error information and describe it in an easily understood way. Users can use these messages to fix the problem by themselves. The error messages will be output to the "errcode.log" file.

The error code is designed to express in three parts, date-time, error code, and explanation. The date-time information could help users differentiate errors in different program executions. The error code part provides information for the pipeline modules and error types. The explanation section displays the most explicit message stating the error/exception and possible solutions.

| PIPELINE         | CODE       | ERROR TYPE                    | EXAMPLES AND ERROR HANDLING  |
|------------------|------------|-------------------------------|--|
| <b>splitMask</b> | SAW-A00001 | Parameters invalid or missing | e.g. "parameters error"<br>Please check your input parameters. Some required parameters might be missed.<br>e.g. "splitBcPos error, expected 1_24 or 2_25"<br>Please check your input CID position is either 1_24 or 2_25. |
|                  | SAW-A00002 | File open failed              | Please check the input file exists and has the correct access permission.  |
|                  | SAW-A00003 | File parse failed             | e.g. "only support .bin or .h5 file."<br>Please check your input file is in the correct file format.   |
|                  | SAW-A00004 | Other IO API error            | e.g. "cannot write to file, /path/to/file"<br>Please check your output directory path is an existing path.   |
| <b>CIDCount</b>  | SAW-A00021 | Parameters invalid or missing | Please check your input parameters. Some required parameters might be missed.  |
|                  | SAW-A00022 | File open failed              | e.g. "cannot open such file, /path/to/file"<br>Please check the input file exists and has the correct access permission.   |
|                  | SAW-A00023 | Failed to parser the file     | Please check your input file is in the correct file format.  |
|                  | SAW-A00024 | Other API error               | Please check Appendix C or contact FAS/FBS for help.   |
|                  | SAW-A00025 | Software exception            | Please check Appendix C or contact FAS/FBS for help.   |

| PIPELINE   | CODE                  | ERROR TYPE   | EXAMPLES AND ERROR HANDLING  |
|------------|-----------------------|--|--|
| mapping    | SAW-A10100            | Parameters invalid or missing  | <p>e.g. "EXITING: FATAL INPUT ERROR: unrecognized parameter name "outSAMAttribute" in input "Command-Line-Initial""</p> <p>Please check the spelling of your argument and parameters.</p> <p>e.g. "please check the umi position and length"</p> <p>Please check the length of the reads in FQ1 are consistent with the parameters set for barcode length and umi length. For example, the length of the sum of barcode and umi set in bcPara file is 35 bp, but one of the reads in FQ1 is 30 bp, then you will see A10101 error code. Please check the parameters set in the bcPara file are consistent with your FQs.</p> |
|            | SAW-A10101            | File open failed   | <p>e.g. "Error, cannot open the file which be expected in gz or ascii format"</p> <p>Please check the file permission and file format.</p> <p>e.g. "barcodePositionMapFile does not exists: /path/to/mask"</p> <p>Please check the mask file exists and has the correct access permission.</p>   |
|            | SAW-A10102            | File parse failed  | <p>e.g. "sequence and quality have different length"</p> <p>Please check the completeness of the reads in FQ. This issue may arise if the FQs are in incorrect format or the file was incompletely written or transferred.</p>   |
|            | SAW-A10103            | Invalid data or data exception   | Error data. Please check the file format and content.  |
|            | SAW-A10104            | File deletion failed   | This error arose if "_STARTmp" directory failed to be deleted. Please check whether the program has completely finished according to the *.Log.progress.out or *.run.log file.   |
|            | SAW-A10105            | File IO failed   | Please contact FAS/FBS for help.   |
|            | SAW-A10106            | Failure on APIs of system and libraries  | Please contact FAS/FBS for help.   |
|            | SAW-A10107            | Software assert  | Please contact FAS/FBS for help.   |
|            | SAW-A10108            | Software exception   | Please contact FAS/FBS for help.   |
| SAW-A10109 | Allocate memory error | This error occurs when you run out of RAM. You may need to kill some jobs that use the same RAM or upgrade your hardware to get more RAM resources for your job. |  |

| PIPELINE          | CODE       | ERROR TYPE                                | EXAMPLES AND ERROR HANDLING   |
|-------------------|------------|---|---|
| <b>mapping</b>    | SAW-A10110 | Out of disk space                         | This error occurs when you store too many files on your hard disk. Please remove some files to free disk space.   |
|                   | SAW-A10200 | Parameter missing                         | Please check your input parameters. Some required parameters might be missed.   |
|                   | SAW-A10201 | CID comparison rate is too low            | This error usually arises because the CID information of the input FQs is not the same as the CID in the input mask file. Please use the correct SN-FQ pairs for mapping. |
|                   | SAW-A10202 | Fail to create the index for the BAM file | Please contact FAS/FBS for help.  |
|                   | SAW-A10300 | Fail to load indexed reference            | Please check the existence, access permission, and completeness for the indexed reference.  |
| <b>mapping-SP</b> | SAW-A12100 | Parameter invalid                         | Please confirm the parameter setting is reasonable. e.g. "cidLen=0"   |
|                   | SAW-A12101 | Parameter missing                         | Please check your input parameters. Some required parameters might be missed.   |
|                   | SAW-A12102 | File open failed                          | Please check the input file that exists and has the correct access permission.  |
|                   | SAW-A12103 | File type invalid                         | Please check the file format.   |
|                   | SAW-A12104 | File parse failed                         | Please check your input file has a valid column number.   |
|                   | SAW-A12105 | Protein name invalid                      | "PIDName" ONLY ACCEPTS letters [a-zA-Z], digits [0-9] and symbols ["(", ")", "-", "_"].   |

| PIPELINE     | CODE       | ERROR TYPE            | EXAMPLES AND ERROR HANDLING  |
|--------------|------------|-----------------------|--|
| <b>count</b> | SAW-A20001 | Parameter missing     | Please check your input parameters. Some required parameters might be missed.  |
|              | SAW-A20002 | File open failed      | Please check the input file exists and has the correct access permission.  |
|              | SAW-A20003 | File parse failed     | Please check your input BAM header is in the correct file format.  |
|              | SAW-A20004 | Allocate memory error | This error occurs when you run out of RAM. You may need to kill some jobs that use the same RAM or upgrade your hardware to get more RAM resources for your job. |
|              | SAW-A20005 | File parse failed     | e.g. "Found <number> gene names with their length exceeding 64 characters"<br>Please check gene names in your input GTF/GFF file.                                |
|              | SAW-A20101 | Parameter missing     | Please check your input parameters. Some required parameters might be missed.  |
|              | SAW-A20102 | File open failed      | Please check the file that exists and has the correct access permission.   |
|              | SAW-A20103 | File parse failed     | Please check your input file has a valid column number.  |
|              | SAW-A20105 | File parse failed     | e.g. "Found <number> gene names with their length exceeding 64 characters"<br>Please check gene names in your input GTF/GFF file.                                |

| PIPELINE        | CODE       | ERROR TYPE                     | EXAMPLES AND ERROR HANDLING   |
|-----------------|------------|--------------------------------|---|
| <b>merge</b>    | SAW-A30001 | Parameter missing              | Please check your input parameters. Some required parameters might be missed.   |
|                 | SAW-A30002 | File open failed               | Please check the input file exists and has the correct access permission.   |
|                 | SAW-A30003 | File parse failed              | Please check your mask file is in the correct file format. Only support .h5/.bin mask file.   |
|                 | SAW-A30004 | Allocate memory error          | Please check whether the range of the coordinates is too large, or you have run out of RAM. You may need to kill some jobs that use the same RAM or upgrade your hardware to get more RAM resources for your job. |
|                 | SAW-A30005 | Fail to open input file        | Fail to open input TXT file. Please check the file that exists and have the correct access permission.  |
| <b>register</b> | SAW-A40001 | Parameter missing              | Please check your input parameters. Some required parameters might be missed.   |
|                 | SAW-A40002 | File open failed               | Please check the input file that exists and has the correct access permission. Or, please check whether a stitched panoramic TIFF (.tif or .tiff) image exists in the TAR.GZ.                                     |
|                 | SAW-A40003 | File parse failed              | Please check your input file is in the correct file format. The -v input gene expression matrix should be either a *tsv, barcode_gene_exp.txt, *.gem.gz, or *raw.gef.   |
|                 | SAW-A40004 | Invalid data or data exception | Error data. Please check the file content. This error may arise because the -v input file is empty, the CZI file in TAR.GZ is invalid, or the QCPassFlag in IPR is 0.   |
|                 | SAW-A40005 | Tissue segmentation error      | Abnormal tissue segmentation reference score in image preprocessing.  |
|                 | SAW-A40006 | IPR field missing              | "Stitch/BGIStitch/StitchedGlobalLoc", does not exist.   |
|                 | SAW-A40007 | Tiled image missing            | Please check the uncompressed image folder has tiled images.  |
|                 | SAW-A40008 | Insufficient GPU memory        | GPU resources in the current node are insufficient. Please redeliver the task on an adequate one.   |

| PIPELINE          | CODE       | ERROR TYPE                     | EXAMPLES AND ERROR HANDLING  |
|-------------------|------------|--------------------------------|--|
| <b>register</b>   | SAW-A40009 | Abnormal chip SN prefix        | Check "ImageInfo -> STOmicsChipSN" in.ipr where it offers the information of chip SN prefix.   |
|                   | SAW-A40401 | Parameter missing              | Please check your <code>imageTools merge</code> input parameters. Some required parameters might be missed.  |
|                   | SAW-A40402 | File open failed               | Please check the <code>imageTools merge</code> input file that exists and has the correct access permission.   |
|                   | SAW-A40405 | Invalid input                  | <code>imageTools merge</code> inputs of less than two images or more than three images.  |
|                   | SAW-A40406 | File pairing failed            | Please check the <code>imageTools merge</code> input TIFF sizes are the same. Since the <code>merge</code> function is used for evaluating segmentation results, the input images are supposed to be the same in size and position (tissue position in the whole image). |
|                   | SAW-A40501 | Parameter missing              | Please check your <code>imageTools overlay</code> input parameters. Some required parameters might be missed.  |
| <b>imageTools</b> | SAW-A40502 | File open failed               | Please check the <code>imageTools overlay</code> input file that exists and has the correct access permission.   |
|                   | SAW-A40504 | Invalid data or data exception | Error data. Please check the file content. This error may arise because the <code>-c</code> input IPR file does not include <code>Stitch/TransformTemplate</code> or <code>Register/MatrixTemplate</code> information.   |
|                   | SAW-A40601 | Parameter missing              | Please check your <code>imageTools img2rpi</code> input parameters. Some required parameters might be missed.  |
|                   | SAW-A40602 | File open failed               | Please check the <code>imageTools img2rpi</code> input file that exists and has the correct access permission.   |
|                   | SAW-A40605 | Invalid input                  | <code>imageTools img2rpi</code> input <code>-i</code> and <code>-g</code> have different length. These two inputs are supposed to be paired.   |
|                   | SAW-A40701 | Parameter missing              | Please check your <code>imageTools ipr2img</code> input parameters. Some required parameters might be missed.  |

| PIPELINE          | CODE       | ERROR TYPE                           | EXAMPLES AND ERROR HANDLING  |
|-------------------|------------|--------------------------------------|--|
| <b>imageTools</b> | SAW-A40702 | File open failed                     | Please check the <code>imageTools ipr2img</code> input file exists and has the correct access permission. Or, please check whether a stitched panoramic TIFF (.tif or .tiff) image exists in the TAR.GZ.   |
|                   | SAW-A40703 | File parse failed                    | Please check your <code>imageTools ipr2img</code> input file is in the <b>ImageStudio</b> out-put TAR.GZ format.   |
|                   | SAW-A40704 | Invalid data or data exception       | Error data. Please check the <code>imageTools ipr2img</code> input IPR file content. This error may arise because the CZI file in TAR.GZ is invalid, or the image has not either automatically or manually registered with the expression matrix. The second circumstance can be confirmed from IPR by checking whether the <code>StereoResepSwitch/register</code> is TRUE (has not performed automatic registration) or the <code>ManualState/register</code> is FALSE (has not performed manual registration). The third possible reason is the <code>StereoResepSwitch/tissueseg</code> is TRUE, therefore cannot output cell segmentation result. |
|                   | SAW-A40706 | File pairing failed                  | Please check the shape of registered tissue segmentation images stored in the <code>imageTools ipr2img</code> input IPR are the same. Or please contact FAS/FBS for help.  |
| <b>tissueCut</b>  | SAW-A50001 | Parameter missing                    | Please check your input parameters. Some required parameters might be missed.  |
|                   | SAW-A50002 | File open failed                     | Please check the file that exists and has the correct access permission.   |
|                   | SAW-A50003 | File parse failed                    | Please check your input file is in the correct file format.  |
|                   | SAW-A50004 | Fail to create output file           | Fail to create output file. Please check your writing permission of the output directory.  |
|                   | SAW-A50005 | Fail to write TIFF                   | Fail to write a TIFF file.   |
|                   | SAW-A50006 | h5AttrWrite error                    | Please check the H5 file attributes.   |
|                   | SAW-A50007 | h5DatasetWrite error                 | Please check the H5 file dataset.  |
|                   | SAW-A50008 | Fail to create TIFF                  | Please check the access permission to write a TIFF image.  |
|                   | SAW-A50009 | Different sizes between TIFF and GEF | Please check the sizes of the TIFF image and GEF (gene expression matrix) respectively. Make sure they are the same size.  |
|                   | SAW-A50010 | Allocate memory error                | This error occurs when you run out of RAM. You may need to kill some jobs that use the same RAM or upgrade your hardware to get more RAM resources for your job.   |



| PIPELINE           | CODE   | ERROR TYPE                       | EXAMPLES AND ERROR HANDLING   |
|--------------------|--|----------------------------------|---|
| <b>cellCut</b>     | SAW-A60001   | Parameter missing                | Please check your input parameters. Some required parameters might be missed.   |
|                    | SAW-A60002   | File open failed                 | Please check the file that exists and has the correct access permission.  |
|                    | SAW-A60003   | File parse failed                | The file does not contain correct information. Please check the file format.  |
|                    | SAW-A60110   | Program version error            | Please check your output GEF version. Your input GEF version might be too old.  |
|                    | SAW-A60111   | Call process error               | e.g. "Please call freeRestriction first, or call restrictRegion function before restrictGene."<br>This error arose because the invocation flow order was messed up. Please modify your invocation flow as prompted. |
|                    | SAW-A60120   | Invalid data or data exception   | Error data. Please check the file content.  |
|                    | SAW-A60121   | File information missing         | Failed to read the file. Please check whether the file is damaged.  |
|                    | SAW-A60122   | File pairing failed              | Please check the TIFF mask size is consistent with the size of expression matrix. Since the mask has been registered with the expression matrix, their sizes are supposed to be the same.                           |
|                    | SAW-A60130   | Fail to create output file       | Fail to create output H5 file. Please check your writing permission of the output directory or contact FAS/FBS for help.  |
|                    | SAW-A60140   | Allocate memory error            | This error occurs when you run out of RAM. You may need to kill some jobs that use the same RAM or upgrade your hardware to get more RAM resources for your job.  |
| SAW-A60150         | Dimensions of gene expression matrix did not match | Please contact FAS/FBS for help. |   |
| <b>calibration</b> | SAW-A60201   | Parameter missing                | Please check your input parameters. Some required parameters might be missed.   |
|                    | SAW-A60202   | File open failed                 | Please check the file that exists and has the correct access permission.  |

| PIPELINE               | CODE       | ERROR TYPE        | EXAMPLES AND ERROR HANDLING   |
|------------------------|------------|-------------------|---|
| <b>spatial-Cluster</b> | SAW-A70001 | Parameter missing | e.g. "-i or --gef_file is missing"<br>Please check your input parameters. Some required parameters might be missed.   |
|                        | SAW-A70002 | File open failed  | e.g. "cannot access /path/to/file: No such file or directory."<br>Please check the file that exists and has the correct access permission.  |
|                        | SAW-A70005 | Value error       | e.g. "The bin size is out of range, please check the range of gef binsize is in [1,10,20,50,100,200,500]."<br>Please reset the bin size as prompted.<br>e.g. "Gene number less than 3000, please check your gef file"<br>Please check the content of your GEF file, and make sure there are at least 3000 genes for clustering. |
| <b>cellCluster</b>     | SAW-A70101 | Parameter missing | e.g. "-i or --gef_file is missing"<br>Please check your input parameters. Some required parameters might be missed.   |
|                        | SAW-A70102 | File open failed  | e.g. "cannot access /path/to/file: No such file or directory."<br>Please check the file that exists and has the correct access permission.  |
|                        | SAW-A70105 | Value error       | e.g. "The bin size is out of range, please check the range of gef binsize is in [1,10,20,50,100,200,500]."<br>Please reset the bin size as prompted.<br>e.g. "Gene number less than 3000, please check your gef file"<br>Please check the content of your GEF file, and make sure there are at least 3000 genes for clustering. |

| PIPELINE          | CODE       | ERROR TYPE                     | EXAMPLES AND ERROR HANDLING   |
|-------------------|------------|--------------------------------|---|
| <b>saturation</b> | SAW-A80001 | Parameter missing              | e.g. "-i is missing."<br>Please check your input parameters. Some required parameters might be missed.  |
|                   | SAW-A80002 | File open failed               | Please check the file that exists and has the correct access permission.  |
|                   | SAW-A80003 | File parse failed              | Invalid GEF file. Please check the file format.   |
|                   | SAW-A80004 | Invalid data or data exception | e.g. "no data left after filter by coordinates."<br>Please check the file content.  |
|                   | SAW-A80005 | Invalid data or data exception | e.g. "total map reads is 0, please check file format from --bcstat"<br>Please check the file content of the input file as prompted.                           |
|                   | SAW-A80006 | File pairing failed            | e.g. "map reads less than annotated reads."<br>Please check the input mapping statistical report and the count statistical report are from the same analysis. |
|                   | SAW-A80007 | Plot error                     | Please contact FAS/FBS for help. PATH environment may not have python3.   |
|                   | SAW-A80008 | Fail to create output          | Fail to generate saturation file, please contact FAS/FBS for help.  |

| PIPELINE         | CODE       | ERROR TYPE                     | EXAMPLES AND ERROR HANDLING   |
|------------------|------------|--------------------------------|---|
| <b>report</b>    | SAW-A90001 | Parameter missing              | e.g. "-m or --barcodeMapStat is missing."<br>Please check your input parameter as prompted. Some required parameters might be missed.   |
|                  | SAW-A90002 | File open failed               | e.g. "cannot access *: No such file or directory."<br>Please check the file that exists and has the correct access permission.  |
|                  | SAW-A90003 | File parse failed              | JSON file format error. This error may arise because the input statistics files were not generated in the same SAW version as <code>report</code> . Or, the input mapping file prefix can not be parsed. Please contact FAS/FBS for help. |
|                  | SAW-A90004 | Invalid data or data exception | e.g. "information loss: fail to find 'bin_[size]' or 'ssDNA' in '*.rpi!'"<br>Please check the file content.   |
|                  | SAW-A90005 | Fail to create output file     | Fail to create output file. Please check your writing permission of the output directory.   |
| <b>report-PT</b> | SAW-A90001 | Parameter missing              | e.g. "-s or --sn is missing."<br>Please check your input parameters. Some required parameters might be missed.  |
|                  | SAW-A90002 | File open failed               | e.g. "cannot access *: No such file or directory."  |
|                  | SAW-A90004 | Invalid data or data exception | e.g. "information loss: fail to find 'bin_[size]' or 'ssDNA' in '*.rpi'."   |
|                  | SAW-A90005 | Fail to write                  | e.g. "failed to write in html" or "failed to write final_result_json."  |

| PIPELINE                    | CODE       | ERROR TYPE                     | EXAMPLES AND ERROR HANDLING  |
|-----------------------------|------------|--------------------------------|--|
| <b>manualReg-<br/>ister</b> | SAW-A40801 | Parameter missing              | Please check your input parameters. Some required parameters might be missed.  |
|                             | SAW-A40802 | File open failed               | Please check the file that exists and has the correct access permission. Or, please check whether the pre-registered image fov_stitched_transformed.tif exists in the input directory.   |
|                             | SAW-A40803 | File parse failed              | Please check your input file is in the correct file format. The -v input gene expression matrix should be either a *tsv, barcode_gene_exp.txt, *.gem.gz, or *raw.gcf.  |
|                             | SAW-A40804 | Invalid data or data exception | Error data. Please check the file content. This error may arise because the -v input file is empty. The second possible reason is that the gene expression matrix information in the IPR Register module (MatrixShape, Xstart, Ystart) does not match with the input GEF file (minX, minY, maxX, maxY), because the manual registration has to be processed on the identical matrix. |
| <b>cellCorrect</b>          | SAW-A13001 | GEF parse failed               | e.g. "-i parameter is invalid, please check your input."<br>Please check the path or file format of GEF.   |
|                             | SAW-A13002 | TIFF parse failed              | e.g. "-m parameter is invalid, please check your input."<br>Please check the path or size of TIFF image.   |
|                             | SAW-A13003 | Fail to create output          | e.g. "-o parameter is invalid, please check your input."<br>Please check the access permission to write files or whether the output directory exists.  |
|                             | SAW-A13004 | Invalid adjusting distance     | e.g. "-d parameter exceeds the range of adjusting distance."<br>Please check whether the adjusting distance is in a proper and reasonable range.   |

| PIPELINE                       | CODE       | ERROR TYPE                     | EXAMPLES AND ERROR HANDLING  |
|--------------------------------|------------|--------------------------------|--|
| <b>multiomics<br/>Analysis</b> | SAW-A14001 | Parameter Invalid              | -r parameter is invalid, please check your Transcriptomics GEM/GEF file. |
|                                | SAW-A14002 | Parameter Invalid              | -p parameter is invalid, please check your Proteomics GEM/GEF file.      |
|                                | SAW-A14003 | Parameter Invalid              | -b parameter is invalid, please check your input binsize.                |
|                                | SAW-A14004 | Parameter Invalid              | -o parameter is invalid, please check your output directory.             |
|                                | SAW-A14005 | Invalid data or data exception | --use_gpu parameter is invalid, please check your input.                 |
|                                | SAW-A14006 | Invalid data or data exception | protein data/rna data is empty, it is probably all filtered out.         |
|                                | SAW-A14007 | Invalid data or data exception | RNA data and protein data are disjoint.                                  |
|                                | SAW-A14008 | Invalid data or data exception | Genes to plot scatters are empty.  |

| PIPELINE         | CODE       | ERROR TYPE                    | EXAMPLES AND ERROR HANDLING  |
|------------------|------------|-------------------------------|--|
| <b>lasso</b>     | SAW-A00031 | Parameters invalid or missing | e.g. "-i/-o/-m/-n is missing"<br>Please check your input parameters. Some required parameters might be missed.                 |
|                  | SAW-A00032 | File open failed              | e.g. "cannot access *: No such file or directory."<br>Please check the file that exists and has the correct access permission. |
|                  | SAW-A00033 | File parse failed             | Ee.g. "file type error: * is not GEF/GEOJSON file."<br>Invalid file. Please check the file format.                             |
| <b>MIDFilter</b> | SAW-A00051 | Parameter Invalid             | Please check your input parameters.  |
|                  | SAW-A00052 | File open failed              | No such file or directory.   |
|                  | SAW-A00053 | File parse failed             | File type error.   |
|                  | SAW-A00054 | Fail to create output         | Generate filtered gef failed.  |

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Please raise GitHub issues for reporting bugs and requesting features:

SAW GitHub Issue Page: <https://github.com/STOmics/SAW/issues>