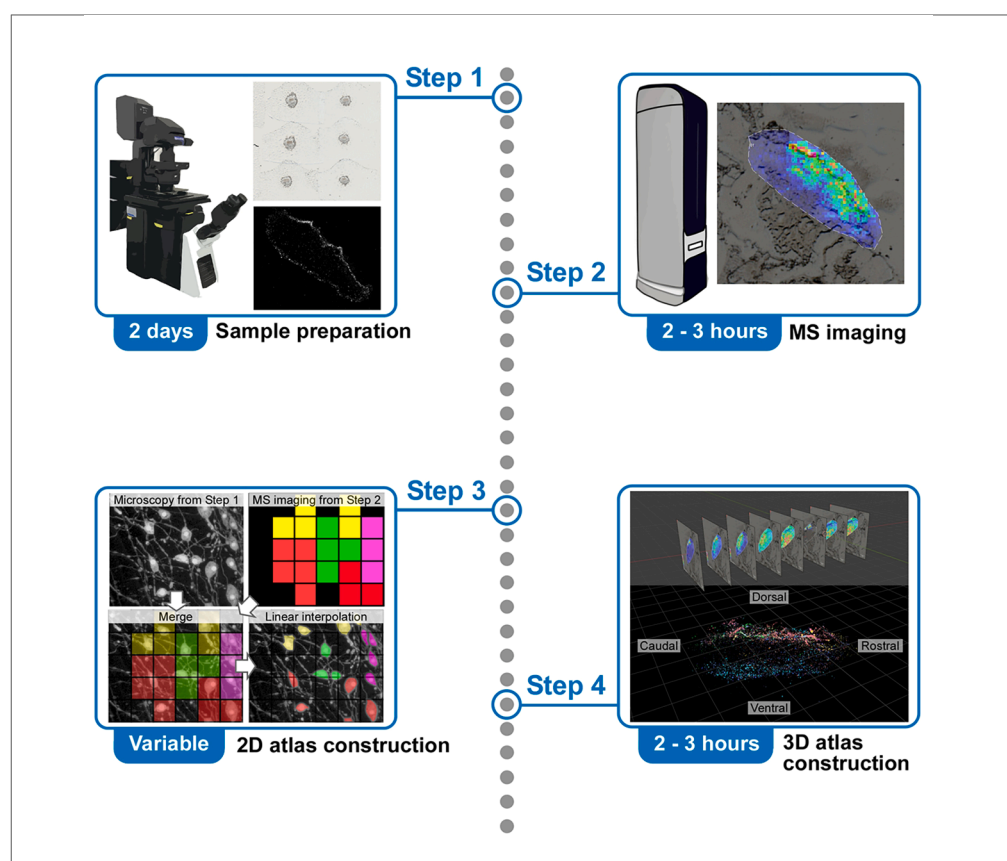


Protocol

Protocol for reconstructing 2D and 3D peptide atlases of the *Ciona* cerebral ganglion at single-cell resolution



The distribution of biomolecules is essential information for understanding biological systems. Here, we present a protocol for 2D and 3D reconstruction of tissue images at the single-cell level by integrating microscopy and mass spectrometry imaging (MSI) data through linear interpolation. We describe steps for sample preparation, MSI, and construction of 2D and 3D single-cell peptide atlases of the cerebral ganglion of *Ciona robusta*. This approach is also applicable to other animals in which target cells are labeled with endogenous fluorescent proteins.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Steps for performing
MSI analysis using the
Ciona cerebral
ganglion

Instructions to
integrate microscopy
and MSI to achieve
single-cell-level
images

2D and 3D
neuropeptide atlas
construction of the
Ciona cerebral
ganglion

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Protocol

Protocol for reconstructing 2D and 3D peptide atlases of the *Ciona* cerebral ganglion at single-cell resolution

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SUMMARY

The distribution of biomolecules is essential information for understanding biological systems. Here, we present a protocol for 2D and 3D reconstruction of tissue images at the single-cell level by integrating microscopy and mass spectrometry imaging (MSI) data through linear interpolation. We describe steps for sample preparation, MSI, and construction of 2D and 3D single-cell peptide atlases of the cerebral ganglion of *Ciona robusta*. This approach is also applicable to other animals in which target cells are labeled with endogenous fluorescent proteins.

For complete details on the use and execution of this protocol, please refer to Osugi et al.¹

BEFORE YOU BEGIN

Mass spectrometry (MS) can simultaneously detect numerous biomolecules including neuropeptides. Among various MS techniques, matrix-assisted laser desorption ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) is particularly versatile and well-suited for label-free and non-targeted analyses, and has provided valuable insights into the distribution of biomolecules and their metabolites. The sensitivity of MALDI-TOF MS was shown to be sufficient for the detection of neuropeptides from isolated single cells.^{2–6} MS imaging (MSI) is a powerful tool for simultaneously detecting and visualizing biomolecules within a single tissue section. MSI provides two-dimensional (2D) images of the spatial distribution of biomolecules, including neuropeptides, in both invertebrates and vertebrates.^{7,8} Three-dimensional (3D) MSI has been developed in earlier studies, enabling visualization of biological molecules in animal organs at spatial resolutions of 100–200 μm .^{9,10} Recent advances in MALDI-MSI have achieved 10 μm lateral resolution.¹¹ In dual-polarity MALDI-MSI methods, 5 μm spatial resolution has been achieved for lipid imaging.¹² These advances highlight the rapid progress of MSI technologies toward cellular and subcellular spatial resolution. 3D MSI has been further advanced to visualize the molecular distributions in the kidney at a resolution of 50 μm .¹³ These studies demonstrate that 2D and 3D MSI have great potential for revealing the spatiotemporal distribution of biomolecules in various organs. Although 2D single-cell MSI have been developing in recent years,^{12,14} single-cell 3D MSI remains challenging.

This protocol describes the step-by-step process of integrating MSI data for peptide distribution and microscopy data for cell distribution using linear interpolation. This process enables us to reconstruct 2D images of peptide distribution at single-cell level. The protocol then describes the process of reconstructing 3D single-cell peptide atlas, in which all 2D data are integrated. The protocol is optimized for transgenic *Ciona robusta* (hereafter referred to as *Ciona*.) expressing Kaede fluorescent protein in



peptidergic neurons.^{15–18} However, this protocol is applicable to other animal tissues in which target cells are labeled with endogenously produced fluorescent protein by transgenic technique. Using this protocol, we constructed 2D and 3D peptide atlas of *Ciona* cerebral ganglion, visualizing the distribution of 23 neuropeptides at single-cell resolution.

Innovation

This protocol advances the visualization of molecular distributions in tissue sections as single-cell resolution images, and enables reconstruction of 2D and 3D atlases of biological molecules within an organ. While recent MSI techniques have enabled visualization of biological molecules in 2D or 3D data,^{7–10} achieving spatial resolution at single-cell level remains challenging. In this protocol, we integrate microscopy images of the cerebral ganglion of *Ciona robusta* in which peptidergic neurons are labeled by an endogenous fluorescent protein, with MSI data acquired at a spatial resolution of 20 μm using custom-developed data processing software. In this software, single-cell images are generated with a combination of established image-processing steps, allowing aggregated MSI signals to be spatially associated with optically defined cellular geometries. The resulting images are then used to generate 2D and 3D atlases of neuropeptide distribution in the *Ciona* cerebral ganglion using commonly available software. The 3D atlas is interactive, and users can rotate the data freely in presentation software, allowing users to explore the molecular distribution from any angle. While the protocol is optimized for the cerebral ganglion of *Ciona robusta*, it is also applicable to other tissues or animals in which target cells are labeled with endogenous fluorescent proteins. For example, transgenic mouse lines expressing GFP under the control of cell specific promoters have been well established, such as astrocyte-specific expression driven by the glial fibrillary acidic protein promoter,¹⁹ although feasibility studies would be required to validate this application in other model animals. In addition, the data-handling workflow is broadly applicable and adaptable to other types of imaging data.

Fluorescent labeling strategy

⌚ Timing: Variable

In this protocol, peptidergic cells are specifically targeted. Because non-fixed tissues are required for MSI analysis, endogenous fluorescent labeling is necessary. To achieve this, a promoter of the prohormone convertase 2 (*PC2*) gene, which is specifically expressed in peptidergic cells, is used in this protocol. Transgenic *Ciona* expressing the fluorescent protein Kaede under the control of the *PC2* promoter are generated and used in MSI analysis. The detailed procedures for generating transgenic *Ciona* are reported in the previous study and in established protocols.^{15,16} In addition, detailed morphological analyses of *PC2* transgenic *Ciona* were reported in our previous studies.^{17,18} The transgenic *Ciona* lines are available through the National BioResource Project (NBRP), Japan (<https://marinebio.nbrp.jp/marinebio/>).

⚠ **CRITICAL:** Identification of a promoter that functions specifically in the target cells is essential for endogenous fluorescent labeling in live tissues.

Tissue preparation for sectioning

⌚ Timing: 30 min to 1 h

1. Dissect the cerebral ganglion from *Ciona* (*PC2*>Kaede line of *Ciona*, Tg[MiCiPC2K]2) and embed the tissue in the cryomold using tissue freezing medium.
2. Modify tissue orientation and rapidly freeze the samples.
 - a. Adjust the direction of the tissues gently with tweezers under a stereomicroscope.
 - b. Freeze the tissues on liquid nitrogen-cooled n-hexane.

⏸ **Pause point:** Samples can be stored at -80°C for up to two weeks.

△ **CRITICAL:** The orientation of tissues (rostral, caudal, dorsal and ventral) is important for reconstruction of tissue images. Carefully record the orientation when embedding the tissue in the medium (Super Cryoembedding Medium (SCEM), Leica Microsystems Japan).

△ **CRITICAL:** Do not use fixatives because they inhibit peptide ionization during MS imaging.

3. Prepare a small piece of paper (approximately 5 mm × 10 mm). Embed it in a mold using tissue freezing medium and freeze it as described above (Figure 1A).

Note: This small piece of paper is used for a mark used for image alignment during MSI analysis. There may be various methods for making, but we recommend using a small paper marker for achieving precise alignment.

Tissue sectioning

⌚ **Timing:** 30 min to 2 h (depending on the number of sections)

4. Cut tissue sections at a thickness 10 µm using a standard cryostat.
 - a. Mount tissue sections on indium-tin-oxide (ITO)-coated glass slides and dry them at 20°C–25°C.
 - b. Dry the sections in a vacuum desiccator for 5 min.

Note: For *Ciona* cerebral ganglion, the chamber and sample holder temperature is set to –18°C.

Note: ITO slide glasses are placed outside the chamber at 20°C–25°C.

Note: A 50-mL centrifuge tube containing silica gel is suitable for storing and transporting sample slides in a dry condition (Figure 1B).

5. Cut sections of the small piece of paper prepared above and place at least three paper sections around the tissue sections on sample slides as alignment markers (Figure 2).

Note: For precise alignment in the MSI analysis, placing alignment markers on the slide glass is important. We recommend using paper sections because their jagged edge provides clear features that are helpful for the precise alignment of sample slides during MSI analysis.

Capturing images

⌚ **Timing:** 1–3 h (depending on the number of sections)

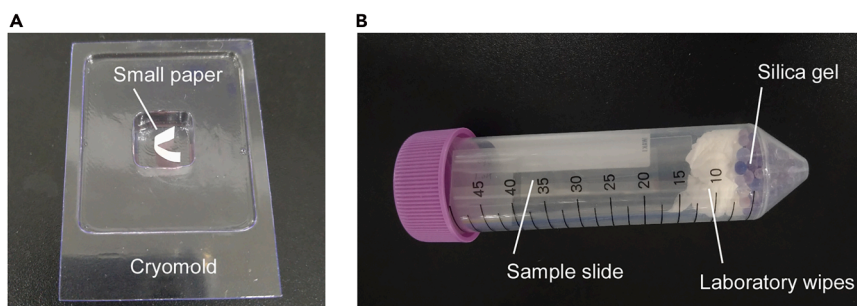


Figure 1. Preparation for tissue sectioning

(A) Preparation of the paper section. A small piece of paper is placed in a cryomold.

(B) Example of a sample storage tube. Silica gel and laboratory wipes are placed at the bottom of a 50-mL tube.

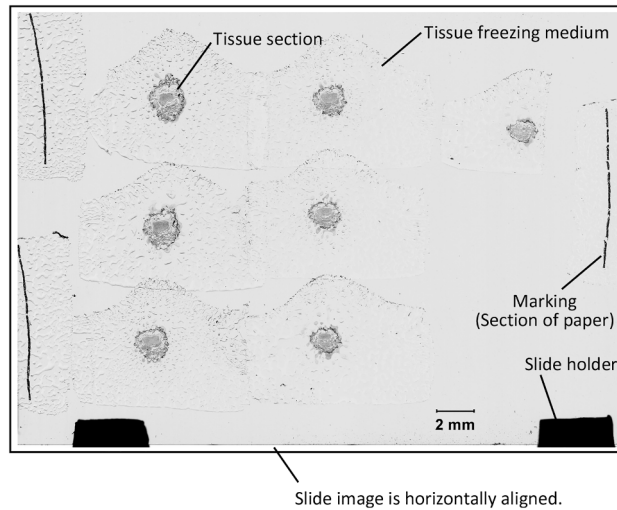


Figure 2. Scanned image of the whole slide

Six tissue sections and three alignment markers are mounted on an ITO-coated slide. The sections are surrounded by tissue freezing medium. This image was captured using the slide scanner described below. Scale bar, 2 mm.

6. Capture fluorescent and differential interference contrast (DIC) images of the tissue samples using a microscope.
 - a. Instrument: Evident (Olympus) FV3000 laser confocal microscope.
 - b. Objective: Olympus UPLXAPO20X/0.80 NA.
 - c. Laser source: 561 nm diode-pumped solid-state (DPSS) laser (OBIS, Coherent).
 - d. Software: Evident FV31SW.
 - e. Scanner and detector: Galvano scanner (one way mode), photomultiplier tube (PMT) detector.
7. Acquisition settings.
 - a. Red channel (Kaede-red): 561 nm excitation, 570–620 nm emission wavelength range, PMT voltage 637 V, Laser transmittance 1.5%.
 - b. DIC channel: PMT voltage 150 V, Laser transmittance 1.5%.
 - c. Gain: 1.0.
 - d. Offset: 0%.
 - e. Sampling speed: 4.0 μ s/pixel.
 - f. Scan size: 1024 $\times$ 768 pixel.
 - g. Pixel size: 0.703 μ m/pixel.
8. Convert fluorescent images to 8-bit images using image-editing software (e.g., GIMP or Photoshop).

Note: 8-bit images are required in the following steps to integrate microscopy images with MSI data.

9. Capture whole-slide images using a slide scanner, as shown in [Figure 2](#).
 - a. Hardware: PrimeHisto XE (Pacific Image Electronics Co., Ltd).
 - b. Software: HistoView v1.00.
 - c. Optical resolution: 10,000 dpi.
 - d. Scan area: 12,965 $\times$ 8649 pixel.
 - e. Color depth: 8 bit.
 - f. Image resolution: 72 pixel/inch.
 - g. Exposure settings: 1 (minimum).
 - h. Auto contrast: On.
 - i. Scan mode: High quality scan.

△ **CRITICAL:** Whole-slide images are essential for performing MSI. The optical resolution of approximately 10,000 dpi is recommended. The resolution of the scanned image obtained by PrimeHisto XE is 72 pixel/inch.

△ **CRITICAL:** Ensure that the image is properly aligned horizontally. If misaligned, correct the orientation using image-editing software. Proper alignment is crucial for accurate image registration during MSI analysis.

△ **CRITICAL:** Check the orientation of the scanned images. Depending on the scanners, the output may appear as a mirror image. Flip the image when necessary.

Note: An expensive device is not required as long as scanning can be performed at a resolution of around 10,000 dpi. A low-cost slide scanner is sufficient. For example, we used the Prime Histo XE slide scanner (Pacific Image Electronics Co., Ltd., New Taipei City, Taiwan), which is available for approximately USD 1,000.

Sample preparation for MSI

⌚ **Timing:** 1–1.5 h

10. Wash the sections with chloroform for 20–25 seconds to remove lipid molecules.
 - a. Immerse the sample slides in a 100-mL flask filled with chloroform for 20–25 seconds.
 - b. After washing, allow them to dry and store them in a centrifuge tube at 20°C–25°C.

Note: Do not move slides during washing.

Note: Perform this step in a fume hood for safety.

11. Prepare the matrix solution.
 - a. Prepare 10 mL of 70% methanol containing 0.1% (v/v) trifluoroacetic acid.
 - b. Add 500 mg of 2,5-dihydroxybenzoic acid (DHB) to the methanol solution and mix thoroughly.

Note: Final concentration of DHB is 50 mg/mL.

Note: The volume of matrix solution is optimized for the auto-sprayer. Adjust the volume as needed depending on the spraying device. Manual airbrush sprayers can also be used.

Note: Matrix selection will vary depending on target molecules. DHB is suitable for peptide detection.

12. Spray matrix solution onto the sample slides.
 - a. Cover both sides of sample slide, including the alignment markers, with Parafilm to prevent matrix spray.
 - b. Place sample slides on the stage and fix them with tape.
 - c. Start the sprayer using following parameters: Liquid flow rate: 0.1 mL/min, Nozzle velocity: 1200 mm/min, Nozzle height: 40 mm, Nozzle temperature: 60°C, Nozzle pass time: 9 counts, Drying time: 0 sec, Track spacing: 2.5 mm, Constant nitrogen flow of 70 kPa.
 - d. After spraying, store the sample slides in the 50-mL centrifuge tube.

Note: Perform this step in a fume hood for safety.

Note: This protocol is optimized for the auto-sprayer (HTX TM-Sprayer; HTX Technologies, LLC, NC). [troubleshooting 1](#).

MSI analysis

⌚ Timing: 2–3 h (depending on the number of samples and MALDI instrument)

13. Confirm that the sample slides, microscopy data (including DIC and fluorescence images), and whole-slide scanned images are ready for use.
14. Apply 0.5–1.0 μ l of peptide standard solution on the sample slide.

⚠ **CRITICAL:** The standard solution should be applied to a peripheral area where matrix solution has been sprayed, but where neither tissues nor tissue-freezing medium are present.

15. Start the MSI analysis according to the manufacturer's instructions. [troubleshooting 2](#).

Note: As the manufacturer's instructions are not publicly available online, key parameters are described as follows: SmartBeam laser operating at 5000 Hz in positive reflectron mode, mass range: m/z 500–4500, smartbeam parameter: single, raster: 20 μ m, scan range; 16 $\times$ 16 μ m (resulting field image 20 $\times$ 20 μ m), laser power; 67, sample rate; 5.0 GS/s, detector gain; 5.0 $\times$, total of 200 laser shots accumulated per pixel.

Note: Laser power varies depending on the instrument. Before starting MSI analysis, confirm that the laser power is sufficient to detect peptides in the tissue.

Note: These parameters are optimized for the rapifleX MALDI-TOF/TOF system (Bruker Daltonics).

⚠ **CRITICAL:** Precise alignment between the scanned image and the tissue position in the instrument is essential for obtaining accurate MSI data. The paper alignment markers prepared in Step 5 in the “before you begin” section facilitates this alignment.

16. Normalize the MSI data by total ion current (TIC).
 - a. Choose a normalize method (TIC) in the software.
 - b. Copy two MSI data files (.ibd and .imzML) and transfer them to your computer.
 - c. Capture screenshots of the tissue image displayed in the MSI software window and save them as scanned images for use in the following steps.

Note: MSI data files are typically large. Ensure that your computer has sufficient disk space for data transfer.

Set up the Python environment

⌚ Timing: 2–3 h

17. Hardware requirements for data processing and generation 2D and 3D atlases.

Set up your computer according to the hardware requirements listed in [Table 1](#) to ensure sufficient processing and visualization of MSI data.

Note: CPU architecture requirements are as follows:

AMD: [GCN 4th gen](#) and newer.

Table 1. Hardware requirements

	Minimum	Recommended
OS	Windows 8.1 (64-bit)	Windows 11
CPU	4 cores	8 cores
RAM	8 GB	32 GB
GPU	2 GB VRAM with OpenGL 4.3, Vulkan 1.3	8 GB VRAM
Storage type	HDD	SSD + HDD

Intel: [Kaby Lake](#) architecture and newer.

Note: Hardware requirements are based on those for Blender, which are sufficient to run the other software used in this protocol. Processing large MSI datasets (~1 GB or larger) using Python scripts may require additional memory for efficient performance. Detailed system requirements for Blender are available on the Blender website (<https://www.blender.org/download/requirements/>).

Note: The hardware configuration used by the authors (desktop computer).

OS: Windows 11,

CPU: Core i5-13500 (14 cores),

RAM: 64 GB,

GPU: NVIDIA GeForce RTX 3060 (12 GB VRAM),

Storage 1: SSD (NVMe, M.2) for the Windows OS,

Storage 2: HDD for experimental data

18. Visit the [Anaconda Installer archive](#). Download and install Anaconda for Windows including python 3.9 (e.g., Anaconda3-2022.10).
19. Launch the Anaconda PowerShell prompt and run the following commands to install the required packages.
 - a. Install OpenCV.

```
(base) PS C:\Users\admin> conda create -n cv39 -c conda-forge python=3.9 "opencv<5" numpy
"blas*=openblas" -y

(base) PS C:\Users\admin> conda activate cv39

(cv39) PS C:\Users\admin> python -c "import cv2, numpy; print('cv2:', cv2.__version__);
print('numpy:', numpy.__version__)"
```

Note: "(base)" indicates the base environment. In the PowerShell prompt, this may appear as "(base) PS C:\Users\admin>" (the "\Users\admin" portion will vary depending on your system). "(cv39)" indicates the Python 3.9 environment, shown as "(cv39) PS C:\Users\admin>". The directory path will be omitted hereafter; refer to "(base)" or "(cv39)" to identify the active environment.

Note: The final command is used to confirm that OpenCV has been installed correctly. Version information (e.g., cv2: 4.12.2 and numpy:2.0.2) will be displayed if the installation is successful.

- b. Install pyimzml.
(cv39) pip install pyimzml

Note: If opencv is not installed, try the following command.

- (cv39) pip install opencv
- c. Install opencv-rolling-ball
(cv39) pip install opencv-rolling-ball.
- d. Install pillow
(cv39) pip install pillow.

20. Download the data processing software ("Imaging MS.zip") from the repository. Unzip the compressed file and place the folder in your preferred directory.

△ **CRITICAL:** Locate the file "tab_sort_files.py" in the folder "Imaging MS program\MS imaging". Modify the file paths in the file according to your local environment. The paths that must be updated are as follows:

```
data.scn_folder.set("C:\\Users\\admin\\desktop\\ImagingMS\\scan").
```

```
data.dic_folder.set("C:\\Users\\admin\\desktop\\ImagingMS\\DIC").
```

```
data.gfp_folder.set("C:\\Users\\admin\\desktop\\ImagingMS\\GFP").
```

```
data.msi_folder.set("C:\\Users\\admin\\desktop\\ImagingMS\\imzML_data").
```

21. In the Anaconda PowerShell prompt, navigate to the "MS imaging" folder and run the program to confirm that the software launches correctly (Figure 3).

```
(cv39) python main.py
```

Note: The installation procedure may change depending on updates from Python package developers. If you encounter errors during installation, refer to the error messages and suggestions shown in the Anaconda prompt and try an alternative approach. [troubleshooting 3](#).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Peptide Calibration Standard II	Bruker Daltonics	Part No:8222570
2,5-Dihydroxybenzoic Acid [Matrix for MALDI-TOF/MS]	Tokyo Chemical Industry	D2933
Tissue freezing medium (Super Cryoembedding Medium)	Leica Microsystems Japan	SCEM
Deposited data		
Tutorial data 1-3	This paper	Zenodo: https://doi.org/10.5281/zenodo.17283391
Experimental models: Organisms/strains		
PC2>Kaede line of <i>Ciona</i> , Tg[MiCiPC2K]2	Sasakura et al. ¹⁵ ; Matsuoka et al. ¹⁶ ; Osugi et al. ¹⁷ ; Osugi et al. ¹⁸	https://doi.org/10.1073/pnas.1230736100 ; https://doi.org/10.1002/gene.20096 ; https://doi.org/10.1371/journal.pone.0180227 ; https://doi.org/10.1038/s41598-020-58884-w
Software and algorithms		
Data processing software	Osugi et al. ¹	Zenodo: https://doi.org/10.5281/zenodo.7297173
Photoshop Elements	Adobe	V20.0

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Image J (Fiji)	Schindelin et al.	https://imagej.net/software/fiji/downloads
GIMP	GIMP's Team	https://www.gimp.org
Blender Long-Term Support (LTS) version	Blender Foundation	https://www.blender.org/
FV31SW	Evident (Olympus)	–
HistoView	Pacific Image Electronics	V1.00
Other		
Tissue-Tek Cryomold	Sakura Finetek Japan Co., Ltd.	#4565
ITO coated glass slide	Bruker Daltonics	8237001
Confocal microscope	Evident (Olympus)	FV3000
Stereomicroscope	Zeiss	SteREO Discovery V8
Cryostat	PHC	CryoStar NX70
Slide scanner	Pacific Image Electronics	Prime Histo XE
Automated matrix sprayer	HTX Technologies	TM-Sprayer
MALDI-TOF MS	Bruker	rapifleX
Personal computer	This paper	See Table 1 and Note

MATERIALS AND EQUIPMENT

DHB matrix solution

Reagent	Final concentration	Amount
2,5-dihydroxybenzoic acid (DHB)	50 mg/mL	500 mg
Methanol	70%	7 mL
Trifluoroacetic acid	0.1%	10 µL
ddH ₂ O	N/A	3 mL
Total	N/A	10 mL

STEP-BY-STEP METHOD DETAILS

Launching the software, processing data, and generating a 2D atlas

⌚ Timing: 1 day–variable (depending on the number of sections and MSI data)

In this step, we integrate MSI data for peptide distribution and microscopy data for cell distribution using linear interpolation in the software and generate a 2D peptide atlas.

1. Download tutorial data 1, which include MSI data. Unzip the archive and place the files into their corresponding folders in the Imaging MS program folder (i.e., DIC, GFP, imzML_data, and scan folders).
2. In the Anaconda PowerShell prompt, navigate to the MS imaging folder and run the program. The interface of the data processing software will appear (Figure 2).

```
(cv39) python main.py.
```

3. Verify that the file paths displayed in the interface match the locations of your data files (Figure 4).
4. Enter the image sizes (size of pixel).

Note: To calculate the pixel size, compare the number of pixels with the physical length indicated by the scale bar in the image. For the tutorial data, the calculations are as follows:

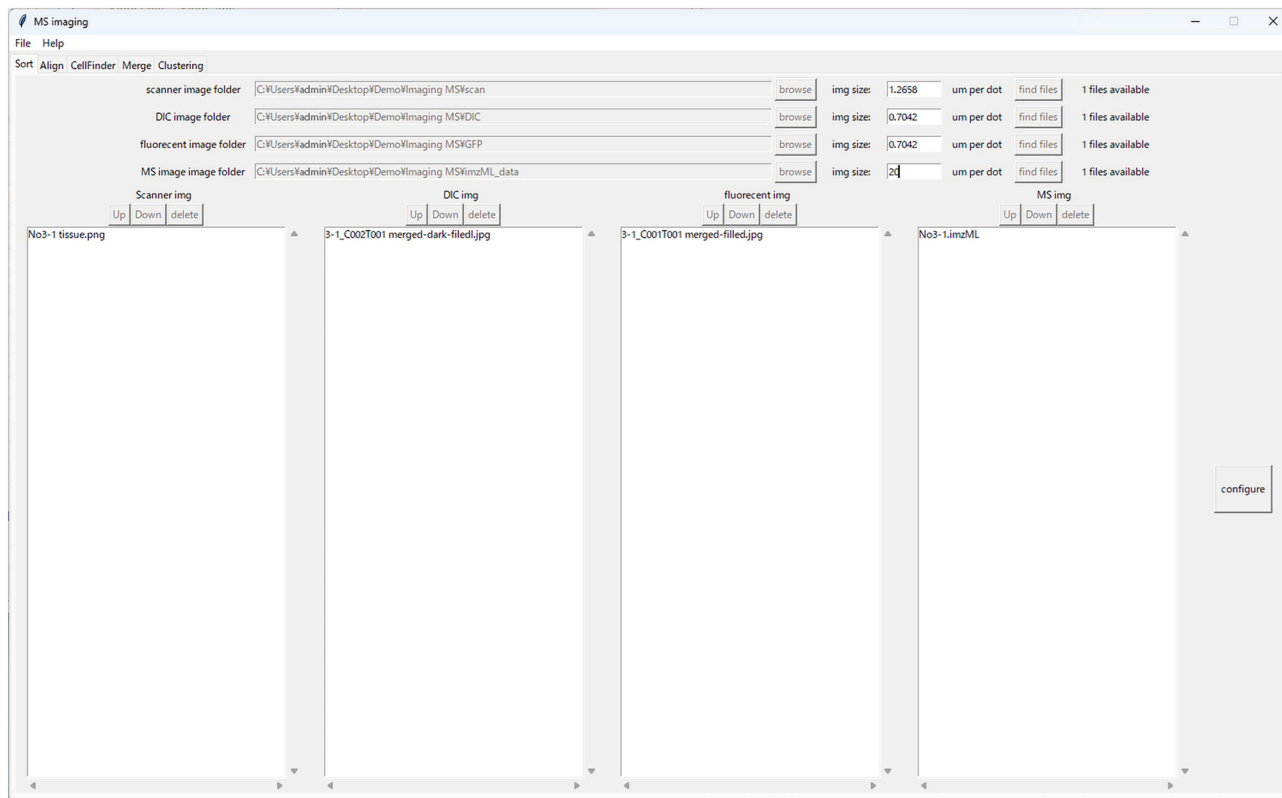


Figure 3. Interface of the data processing software

This interface appears when the Python environment has been configured correctly.

Microscopy image:

71 pixels = 50 μm .

1 pixel = 0.7042 μm .

Scanned image:

79 pixels = 100 μm .

1 pixel = 1.2658 μm .

5. Click the "find files" button. A "success" window will appear once the data have been loaded successfully. Click the "OK" button to proceed.
6. After clicking all "find files" buttons, verify the loaded data and then click the "configure" button.

Note: The number of data points must be identical across all windows.

7. Navigate to the Align tab (Figure 5).
 - a. Select the data in the data selection window.

Note: The corresponding image will appear in the main window.

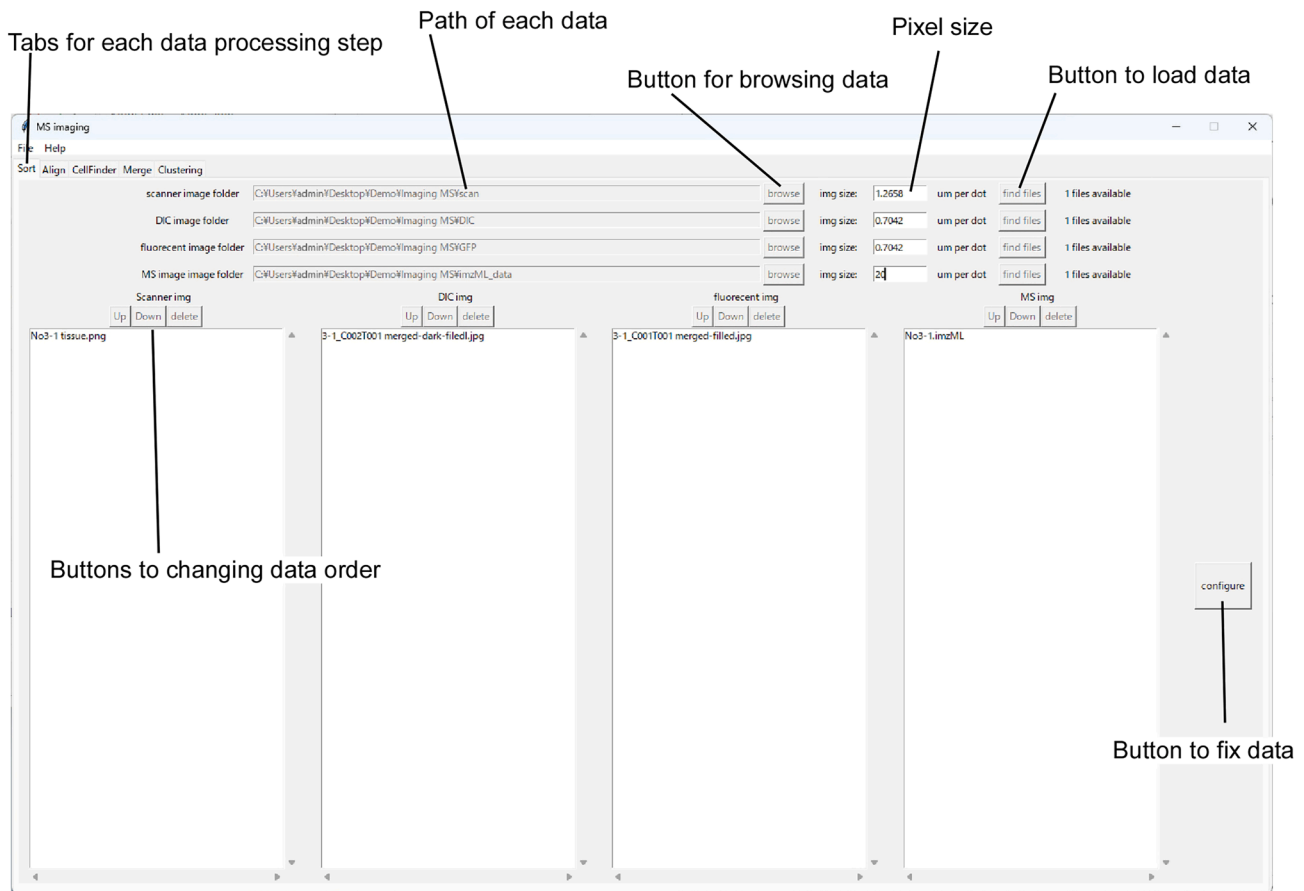


Figure 4. Sort tab interface of the data processing software

Data paths can be entered manually or selected using the “browse” button. Image size can be entered directly. The “find files” button loads the data, and the loaded files are listed in the window. The “configure button” locks the data for the next steps. Each data-processing function is accessible through the tabs.

- b. Adjust the DIC image to match the scanned image by entering the DIC image coordinates (x, y) and rotation angle.
- c. Adjust the MSI area to cover the MSI ROI, indicated by black dotted line, by entering the MS image coordinates (x, y).

Note: The aligned image is shown in [Figure 6](#).

△ CRITICAL: Carefully align DIC and scanned images based on the outline of tissue sections. Misalignment can substantially affect atlas quality.

8. Navigate to the CellFinder tab ([Figure 7](#)).
 - a. Compare the black-and-white image in this tab with fluorescent image.
 - b. Adjust the threshold value until the cell images are clearly visible.

Note: The resulting fitted image is shown in [Figure 8](#).

9. Navigate to the Merge tab ([Figure 9](#)). Select the appropriate methods and specify the parameters as follows.
 - a. Select dataset in the data selection window.

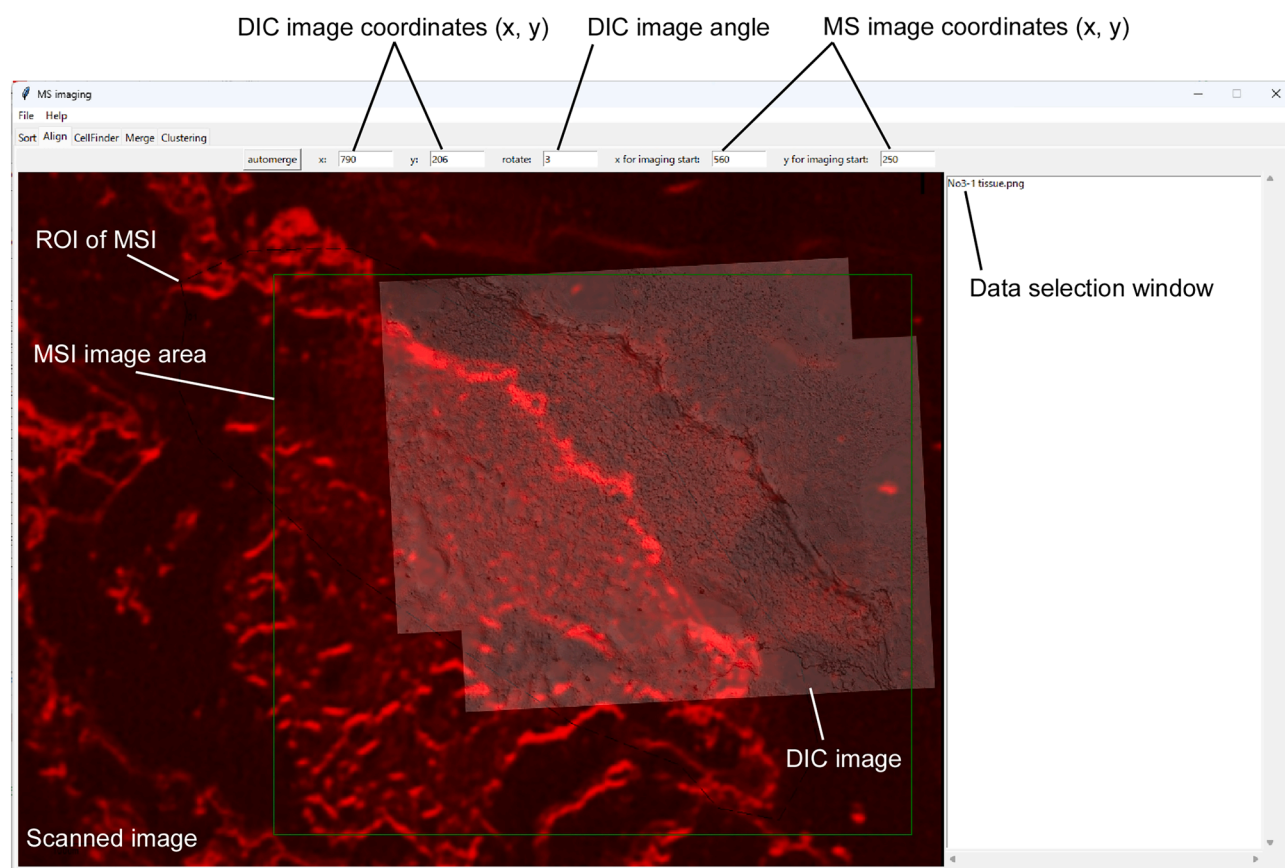


Figure 5. Align tab interface of the data processing software

The red image represents the scanned image, and the translucent image represents the DIC image. The MSI image area is shown as a green box, and the MSI ROI is indicated by the black dotted line. The DIC image can be repositioned by entering DIC image coordinates (x, y) or rotation angle. The MSI area can be repositioned by entering the MSI image coordinates (x, y). The position of the scanned image is fixed.

- b. Select the interpolation method.
 - i. **Linear:** Interpolates pixel values using linear weighting of the nearest neighboring pixels (typically a 2×2 area).
 - ii. **Cubic:** Applies cubic convolution using 16 neighboring pixels (4×4 area) to generate smoother transitions.
 - iii. **Lanczos:** Applies a sinc-based filter over a wider neighborhood (e.g., 8×8 area) to preserve fine details with high accuracy.
- c. Select the filter method. In most cases, choose "by cell region".
- d. Set mass range of the target peptides and click the "update" button.
- e. Click the "Auto max" button to automatically set the maximum intensity threshold so that the signals become clearly visible.

Note: The "single value/single cell" option is disabled in the current version.

Note: Clicking the "Auto max" button automatically enters the maximum intensity for each peptide into the corresponding "max" fields. The 99th percentile of pixel intensity values is used to exclude outlier noise and define the upper intensity limit for image normalization. Maximum values can also be adjusted manually if necessary. Clicking the "update" button refreshes the image after modifying maximum values or mass ranges.

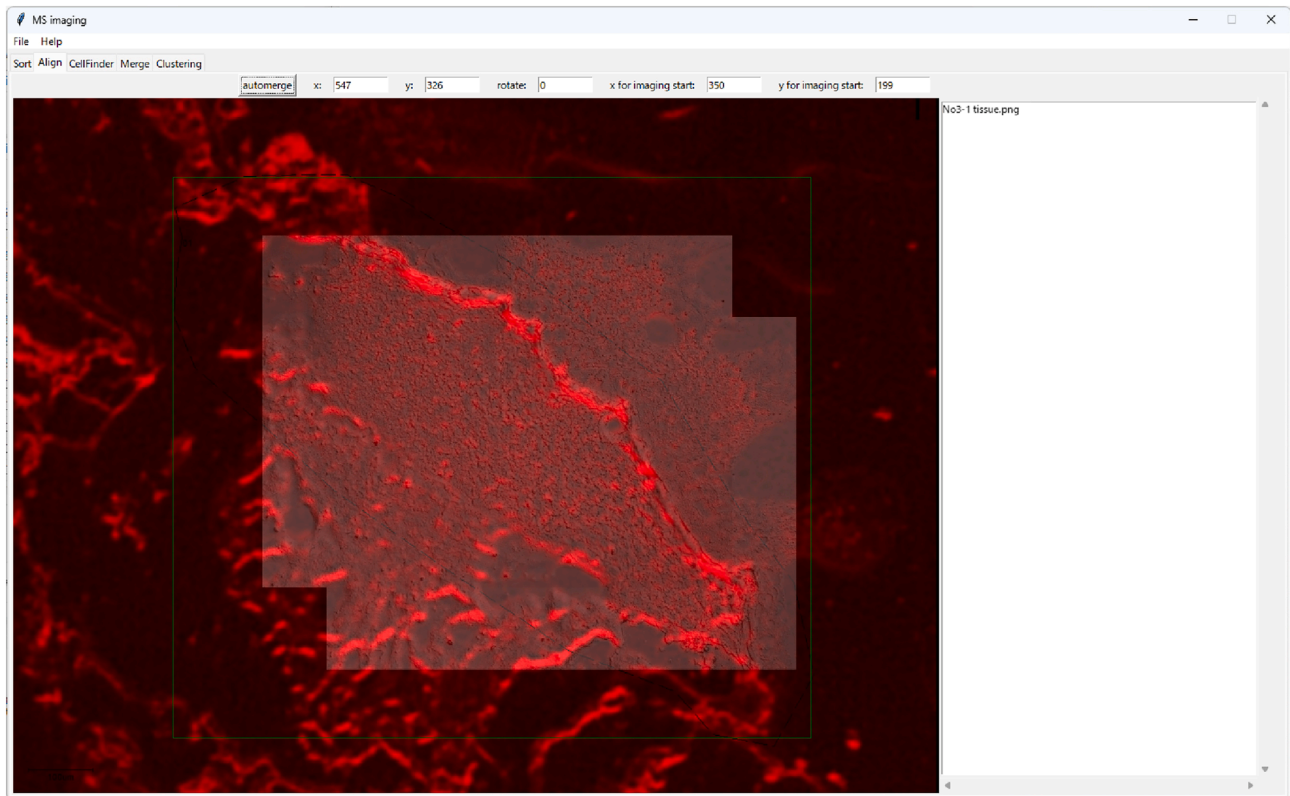


Figure 6. Aligned images in the Align tab

The DIC image is fitted to the scanned image after entering the DIC image coordinates (x, y), rotation angle, and MSI image coordinates (x, y).

10. Collect single-color images for each peptide without merging them with the DIC or scanned Images for use in 2D and 3D atlas construction (Figure 10).

Note: Use the screen shot function and save images as PNG files. Create one additional image merged with the DIC image as a reference of the microscopy area. In the example shown in Figure 10, the m/z 1158.6584–1158.8584 signal is displayed as a red image, with or without DIC image. Large values (e.g., 400) are entered for the green and blue channels to eliminate their colors and obtain a red image.

11. Load all single-color images for each peptide as separate layers in image-editing software (e.g., GIMP or Photoshop).
 - a. Duplicate each layer to generate three color versions (red, green, and blue), which allows you to switch the displayed color for each peptide easily.
 - b. Modify each color channel (red, green, and blue) using channel mixer function of the software (Figure 11).

Note: A two-dimensional atlas can be constructed by combining these three-color layers.

Construction of a 3D atlas

⌚ Timing: 1 day

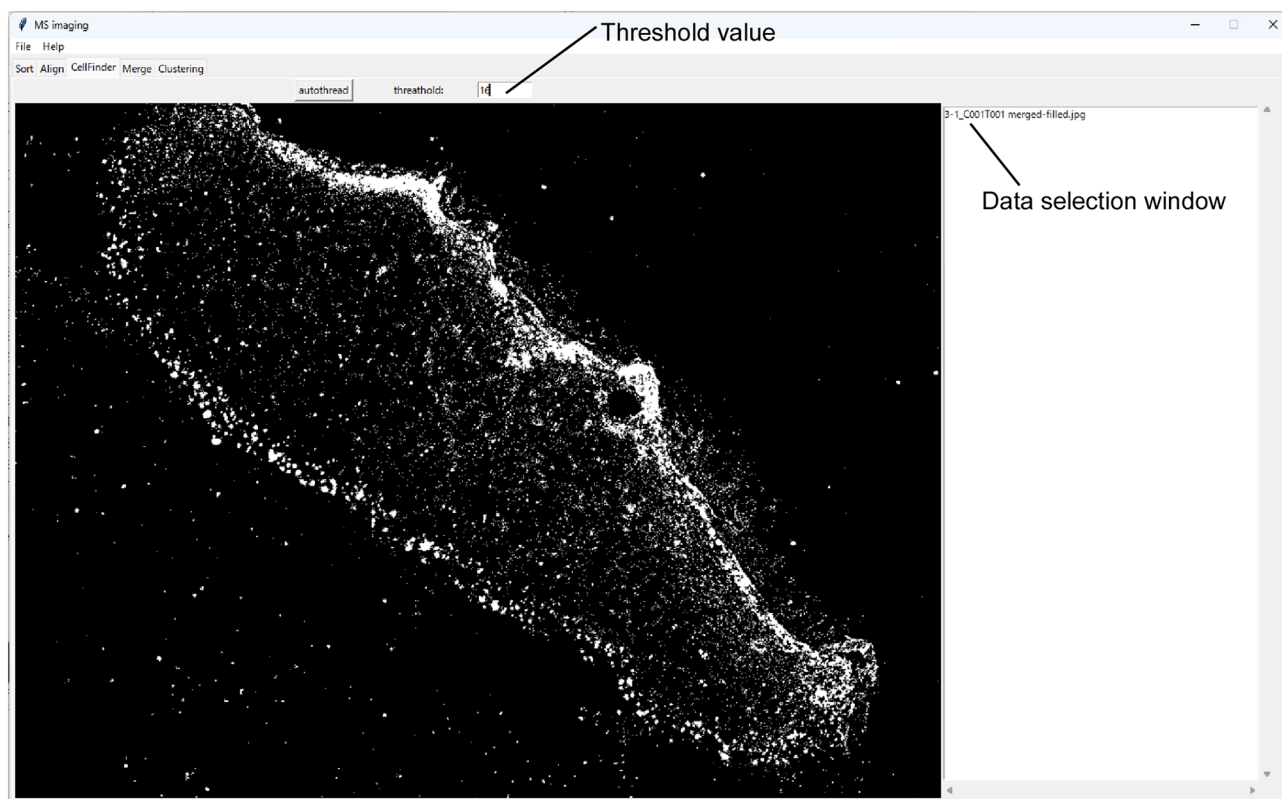


Figure 7. CellFinder tab of the data processing software

The fluorescence image is displayed as black-and-white image. Increasing the threshold value reduces background noise. The “auto threshold” button is disabled in the current version because the program is a prototype.

In this step, we integrate all 2D data generated in the above steps and construct a 3D peptide atlas using multiple software.

12. Download tutorial data 2, which includes three image sets and an image for scale.
13. Open the image set of each mass value (e.g., *mz*787 image set) and set parameters in Image J (Fiji).
 - a. Open “Image for scale.jpg” and set the scale based on the scale bar in the image.
 - b. Use the Straight line tool to trace the scale bar.
 - c. Select “Set scale” from the Analyze menu (Figure 12A).
 - d. Enter “Known distance” and “Unit of length”.
 - e. Check the “Global” check box.
 - f. Select “Properties” from the Image menu (Figure 12B).
 - g. Set the voxel depth to the thickness of the sections (e.g., 8 μm).
 - h. Check the “Global” check box and click the “OK” button.

Note: The thickness can be adjusted to improve the visibility of the 3D data rather than to match the exact physical thickness of the sections.

△ CRITICAL: The pixel information (e.g., 0.7867 pixels/ μm in Figure 12A) and pixel width (e.g., 1.2658 μm in Figure 12B) must be identical across all datasets for constructing the 3D atlas. Any differences in these values will cause inconsistencies in coordinate positions within the 3D data.

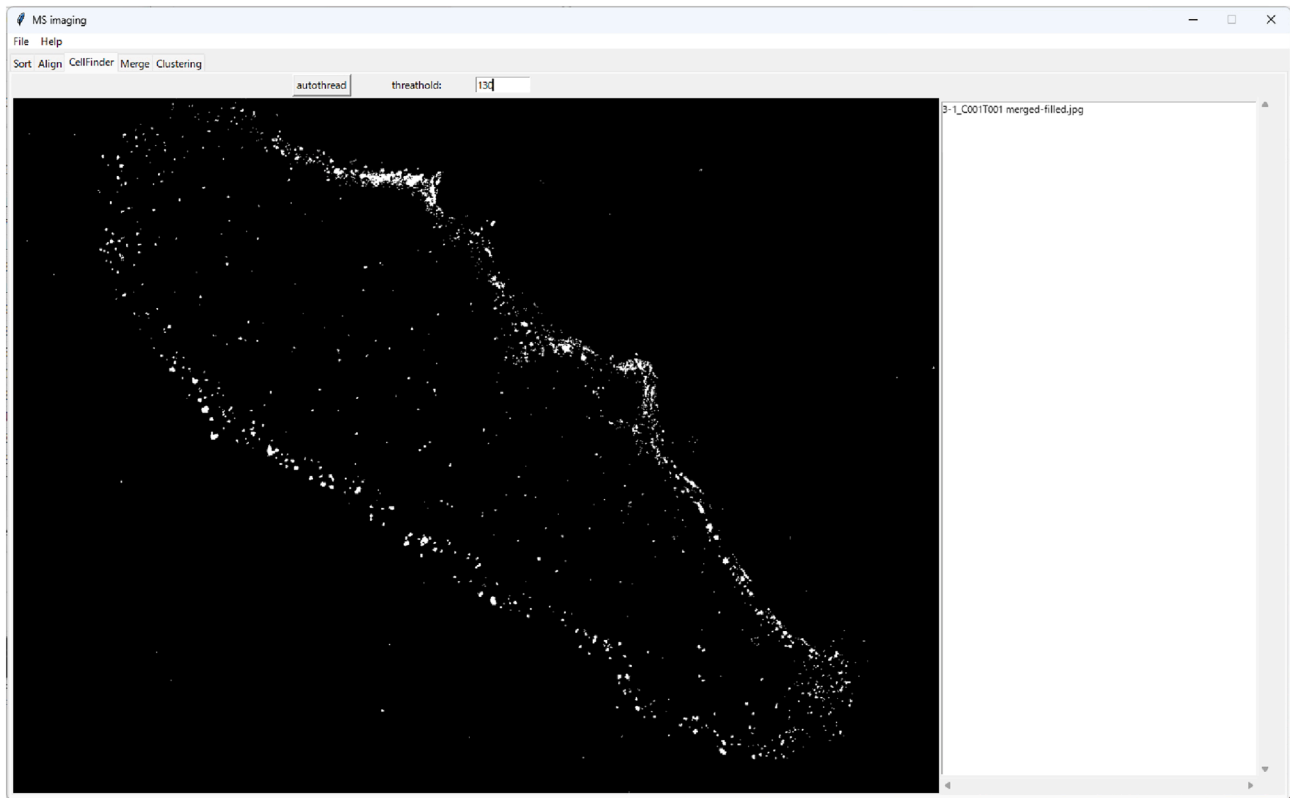


Figure 8. CellFinder tab after threshold adjustment

The cell images are clearly visible.

14. Generate image stacks and create a 3D view.
 - a. Select "Images to Stack" from the Stacks submenu in the Image menu.
 - b. Select "3D Viewer" from the Plugins menu and configure the parameters (Figure 13A).
 - c. Choose "Surface" in the Display as menu.
 - d. Select a single color (e.g., Red).
 - e. Set the Resampling factor to 1.
 - f. Inspect the 3D image in the 3D Viewer window (Figure 13B).
 - g. Export the surfaces as STL (ASCII) or STL (binary) file.

Note: Both ASCII and binary formats work in the following steps. In this manuscript, the ASCII format is used.

15. Download tutorial data 3, which includes the STL files. Import the STL files into Blender (LTS version) to construct 3D atlas.
 - a. Launch Blender and delete the default cube object. Then import the STL files (Figure 14A).
 - b. Zoom out and inspect the 3D data. Select one layer and create a new material for it (Figure 14B).
 - c. Click the color bar in the "Base Color" and choose a color in the color chart. Select "Viewport Shading" to visualize the colored 3D data (Figure 15A).
 - d. Create materials for all remaining 3D datasets (Figure 15B).

Note: Ensure that the Blender is set to "Object mode" (Figure 14B). [troubleshooting 4](#).

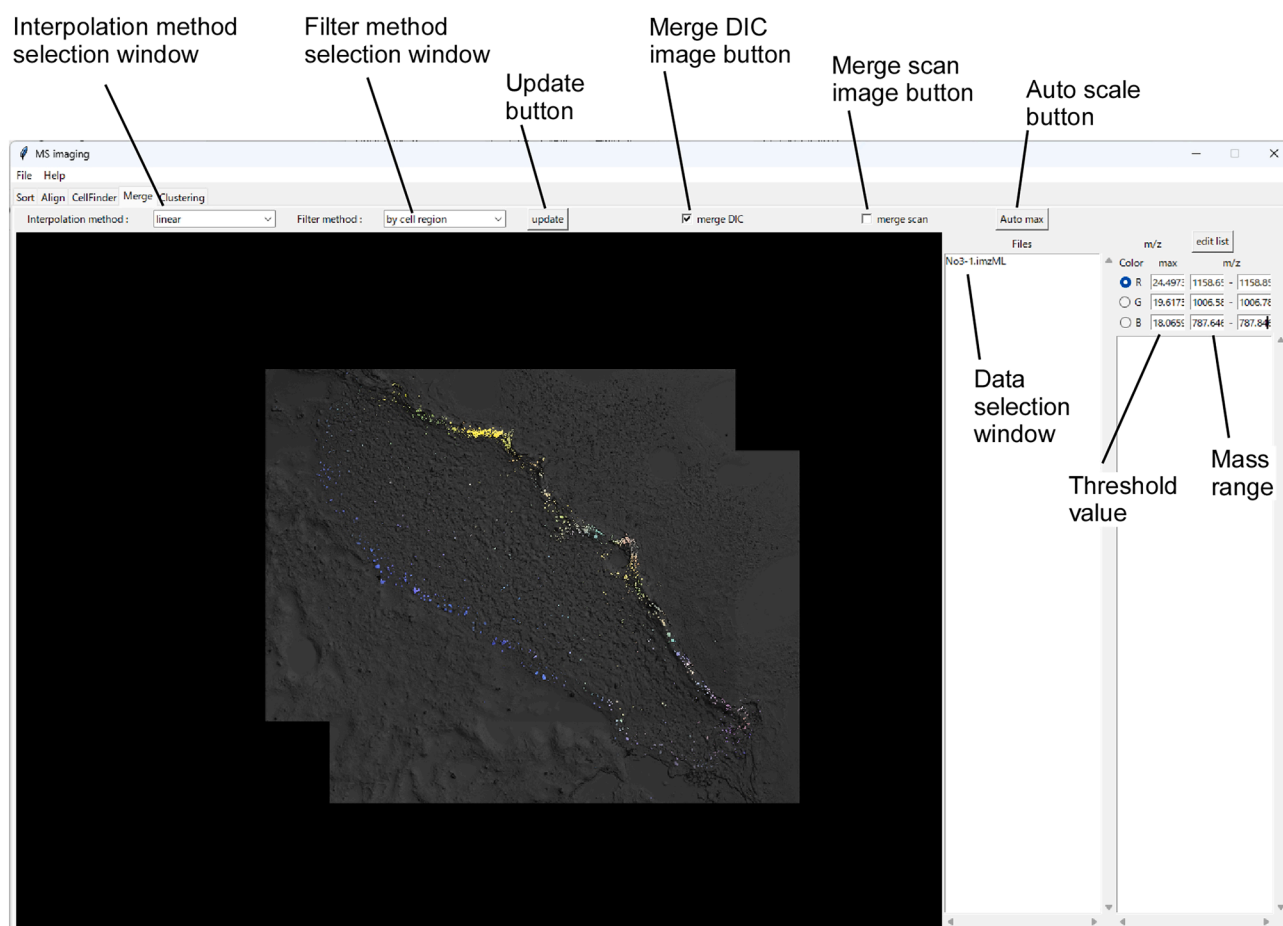


Figure 9. Merge tab of the data processing software

In this window, MSI data are merged with microscopy images using the interpolation method. Up to three mass peptide masses can be visualized simultaneously. The “edit list” button is disabled in the current version.

16. Adjust the coordinates of the 3D data according to their anatomical orientation.

Note: The orientation of the 3D data should match the orientation of the original tissue.
[troubleshooting 5](#).

17. Select all 3D datasets of interest and export them in the glTF 2.0 format ([Figure 16A](#)).

Note: The resulting.glb file can be inserted into PowerPoint presentation via drag-and-drop ([Figure 16B](#)). The 3D viewer application available for Windows can also open.glb files.

EXPECTED OUTCOMES

The present protocol enables users to construct 2D and 3D atlases of MSI data at single-cell resolution ([Figures 11](#) and [15](#)). Tutorial datasets for each step are provided through the Zenodo repository.

Prior to beginning atlas construction, users perform MSI analysis following the standard procedures described in the “[before you begin](#)” section. This yields MSI data with a spatial resolution of 20 μm . Careful manipulations and settings in Steps 5, 9 and 15 are essential for obtaining high-quality MSI

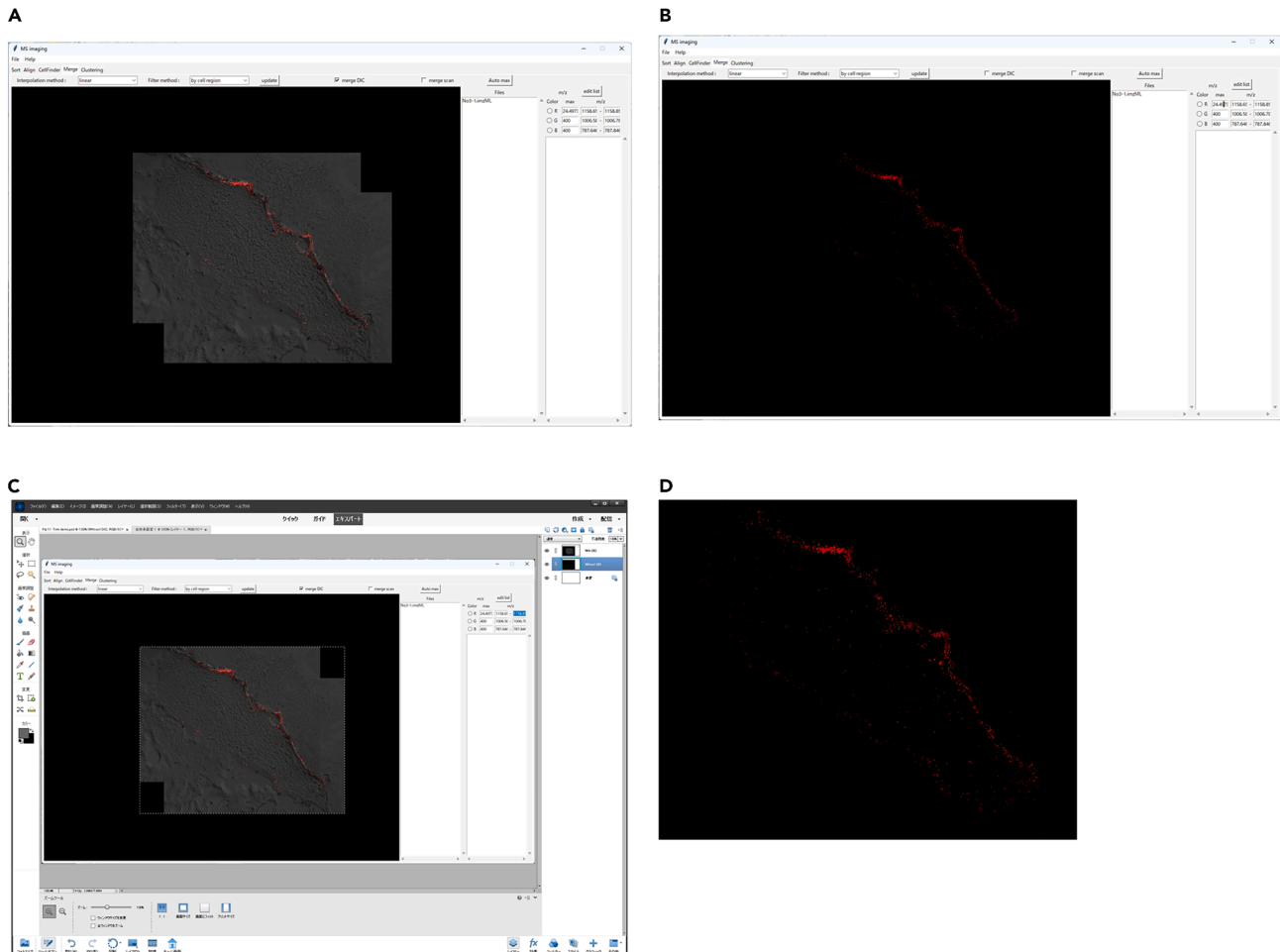


Figure 10. Image process for trimming

- (A) Screenshot of the peptide distribution image with the DIC image.
- (B) Screenshot of the peptide distribution image without the DIC image.
- (C) The two images in (A) and (B) displayed as layers in an image-editing software (Photoshop elements).
- (D) The screen shot of the peptide distribution image without DIC image trimmed according to the DIC image area.

data. After MSI analysis, users set up a Python environment to run the data processing software, which converts MSI data into single-cell-resolution images.

In the main protocol, microscopy images and MSI data are integrated using the provided software in conjunction with the tutorial dataset. The final output is a set of 2D and 3D atlases visualizing the distribution of neuropeptides in the *Ciona* cerebral ganglion. Although the tutorial dataset originates from *Ciona* cerebral ganglion, the protocol is also applicable to other animal species, if target cells are labelled with endogenous fluorescent proteins.

LIMITATIONS

This protocol requires labeling target cells with endogenous fluorescent proteins. Because MSI analysis is generally difficult to perform on fixed tissues, the available labeling methods are limited.

The quality of the resulting atlases depends on both the resolution of the microscopy images and the signal intensity of the MSI data. Accurate alignment of tissue images during MSI analyses and data processing is also essential, as misalignment can substantially reduce atlas quality. In addition,

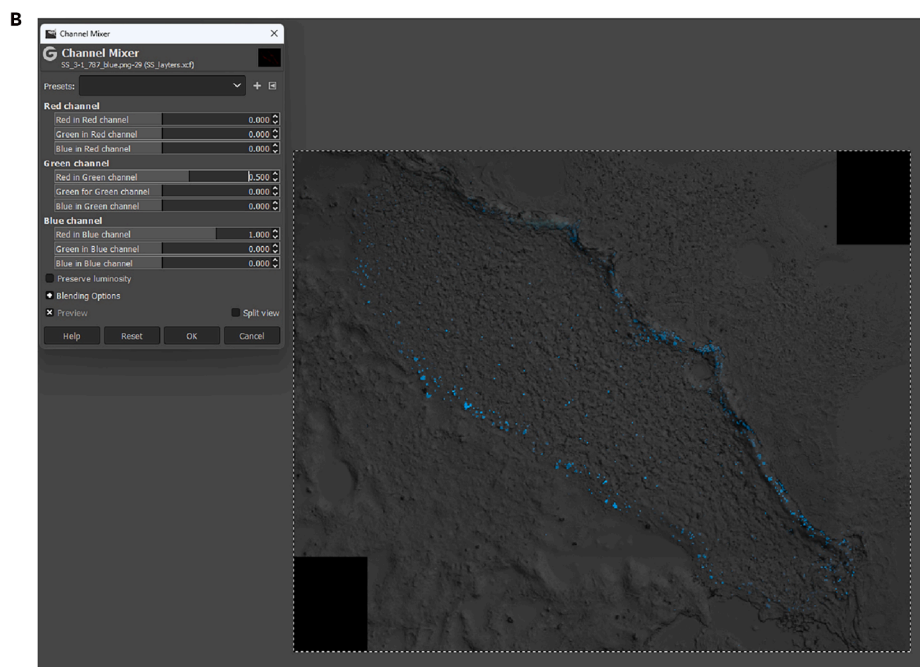
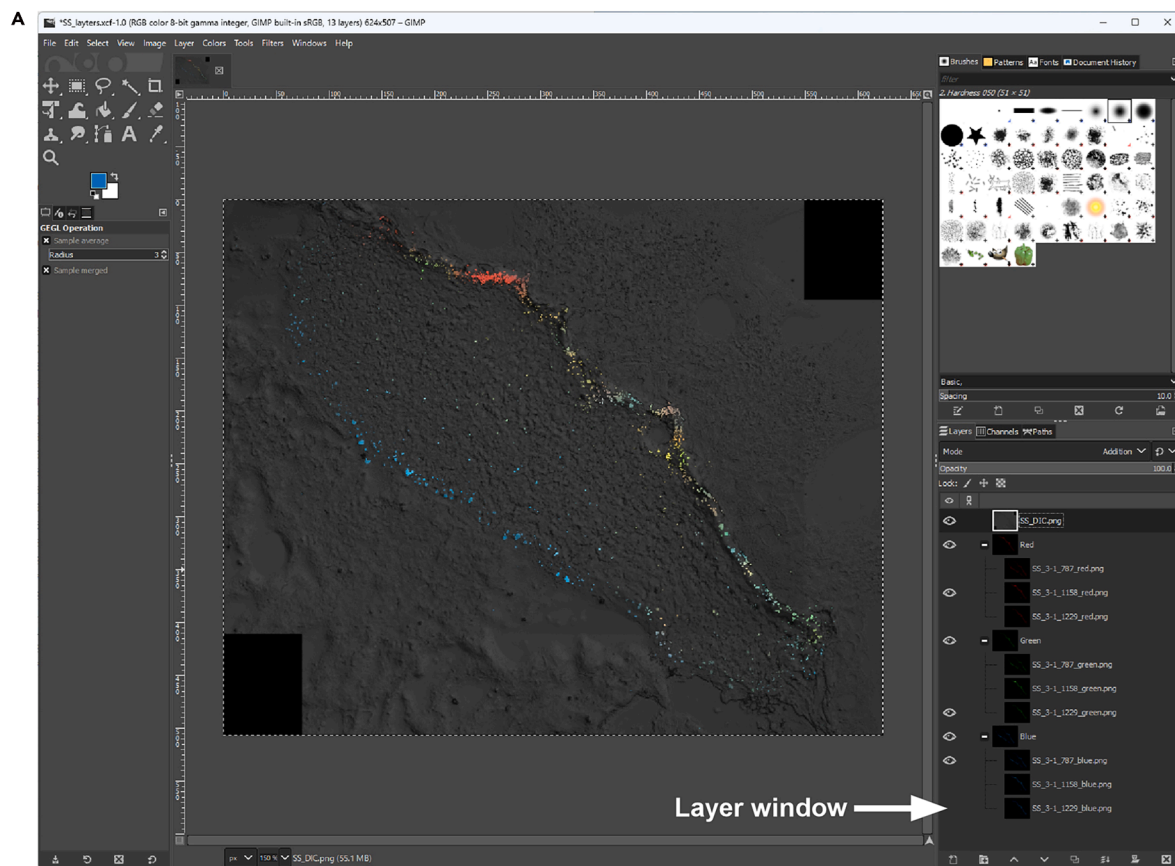


Figure 11. Example of layer images in an image-editing software (GIMP)

(A) Overview of the user interface. The layer window is shown in the lower-right corner.

(B) The blue channel is adjusted to a lighter blue to improve visibility to the human eye, as shown in the parameter window.

the accuracy of the generated atlases depends on both cell size and spatial resolution of MSI. When the MSI pixel is larger than cell size, it is difficult to assign the original MSI signals to closely localized individual cells. This limitation may affect the counting of specific peptidergic cells, potentially leading to slight overestimation under such conditions. As a result, precise quantitative analysis of cell numbers expressing specific peptides remains challenging in the tutorial dataset. Advances in MSI instrumentation that enable smaller acquisition pixel sizes with enough detection sensitivity may further enhance the spatial correspondence between MSI signals and cellular structures in this workflow.

The workflow relies on multiple software tools. The Python environment may require future updates if installation errors arise, and users may need to adjust the setup accordingly. In addition, the computer hardware should meet the performance requirements of the software, particularly for handling and manipulating 3D data.

TROUBLESHOOTING

Problem 1

Matrix auto-sprayers are not available.

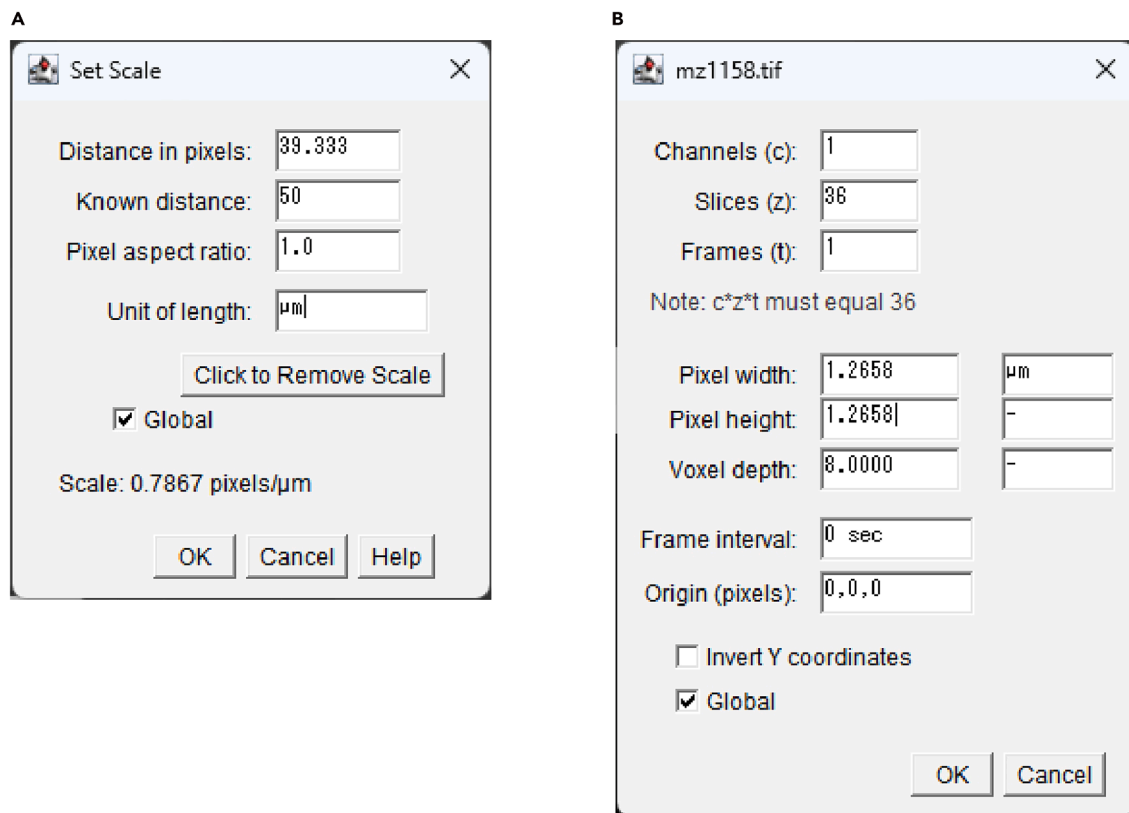


Figure 12. Parameter windows of Image J (Fiji)

(A) Set Scale window. A known distance of 50 µm is entered, and the unit of length is set to "µm" (type "u" instead of "µ").

(B) Properties window. A section thickness of 8 µm is entered in the Voxel depth field.

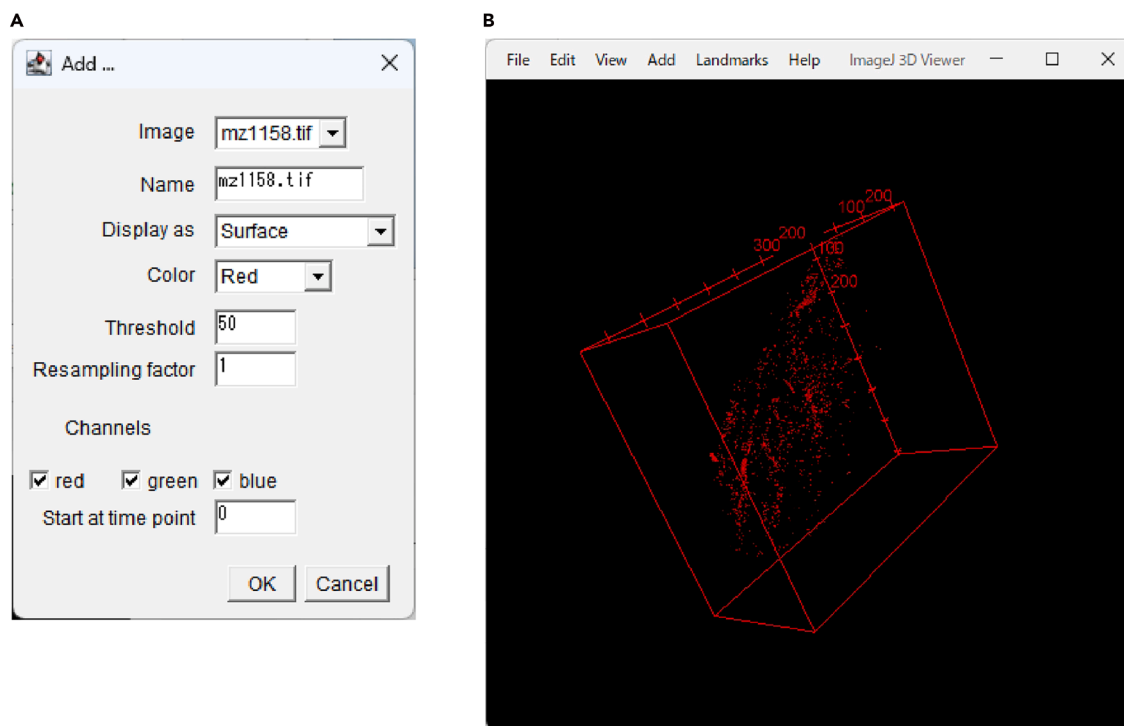


Figure 13. 3D Viewer window of Image J (Fiji)

(A) Parameter settings. "Surface" is selected in the Display as menu, and Resampling factor is set to 1.

(B) 3D Viewer window displaying the interactive 3D image. Use the Export surfaces menu in this window to export an STL file.

Potential solution

Manual airbrushes designed for hobby use can also be used for matrix spraying. Prepare an airbrush handpiece and an air compressor. An airbrush handpiece with high solvent resistance is recommended. Perform this step in a fume hood for safety.

Problem 2

Acquisition of peptide signals is difficult in MSI analysis.

Potential solution

- The freshness of tissue sections is critical. Long-term storage of tissue sections can result in weak signals, even when sections are stored at -80°C . Tissue sectioning and MSI analysis are therefore recommended to be performed seamlessly.
- Confirm the washing conditions in step 10 of the chapter "Sample preparation for MSI". Insufficient washing can result in the preferential detection of lipid molecules rather than peptides.
- Confirm the condition of the matrix applied on tissues. Uneven matrix coating can affect MSI signal acquisition.
- Adjust the laser power to a higher setting. In our experiments, the laser power was set to 60%–70% on the repiflex MALDI-TOF/TOF system.

Problem 3

The data processing program does not work.

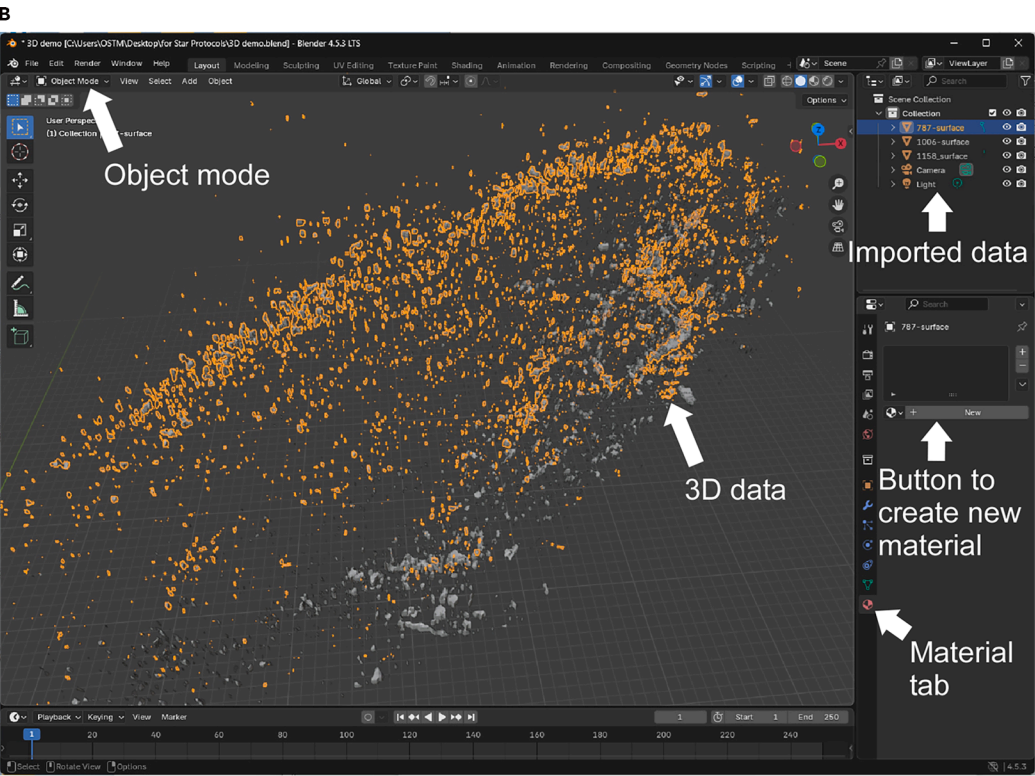
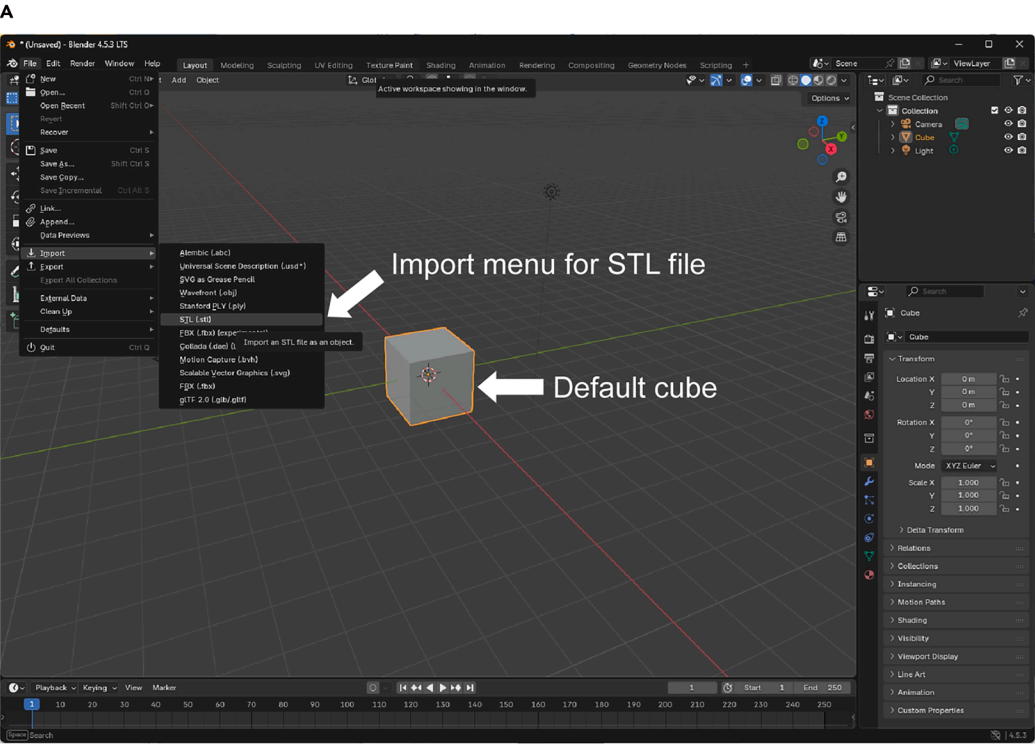


Figure 14. Initial interface of Blender and imported 3D data

(A) The default cube and the import menu are shown.

(B) The imported 3D data displayed in the main window and listed in the Scene Collection window. The “Material” tab is used to apply pseudo color to the 3D data.

Potential solution

- Confirm the Python version. In this protocol, Python 3.9 is recommended.
- Confirm the paths specified in the file “tab_sort_files.py” located in the folder “Imaging MS program\MS imaging”. Open this file in a text editor and modify the paths according to your local environment, as shown in step 20. In Windows, file paths generally include the username. For example, if the username is “STAR” and the folder is located on the Desktop, the path should be “C:\Users\STAR\Desktop\Imaging MS\scan”.
- Confirm that all data are correctly set in their respective folders.

Problem 4

The 3D data is no longer rendered when it is positioned too far from the viewpoint in Blender.

Potential solution

Open the sidebar and select the “View” tab. Increase the value of the “End” parameter (Figures 17A and 17B).

Note: The sidebar can be shown or hidden from the View menu.

Problem 5

The coordinates of the 3D data do not align with the X-Y axes in Blender.

Potential solution

Select all 3D datasets, then choose “Move” or “Rotate” from the tool bar. Use the X, Y, and Z axis arrows to translate the objects or use the rotation sphere to adjust their orientation (Figures 18A and 18B).

RESOURCE AVAILABILITY

Lead contact

Further information and requests should be directed to and will be fulfilled by the lead contact, Tomohiro Osugi (osugi@sunbor.or.jp).

Technical contact

Technical questions about this protocol should be directed to the technical contacts, Akira Shiraishi (shiraishi@sunbor.or.jp) and Tomohiro Osugi (osugi@sunbor.or.jp).

Materials availability

The transgenic *Ciona* line used in this study is available from the National BioResource Project in Japan (<https://marinebio.nbrp.jp/marinebio/>).

Data and code availability

- Tutorial data are available in the Zenodo database (<https://doi.org/10.5281/zenodo.17283391>).
- The data processing software is available in the Zenodo database (<https://zenodo.org/records/7297174>).
- Data and software are also listed in the [key resources table](#).

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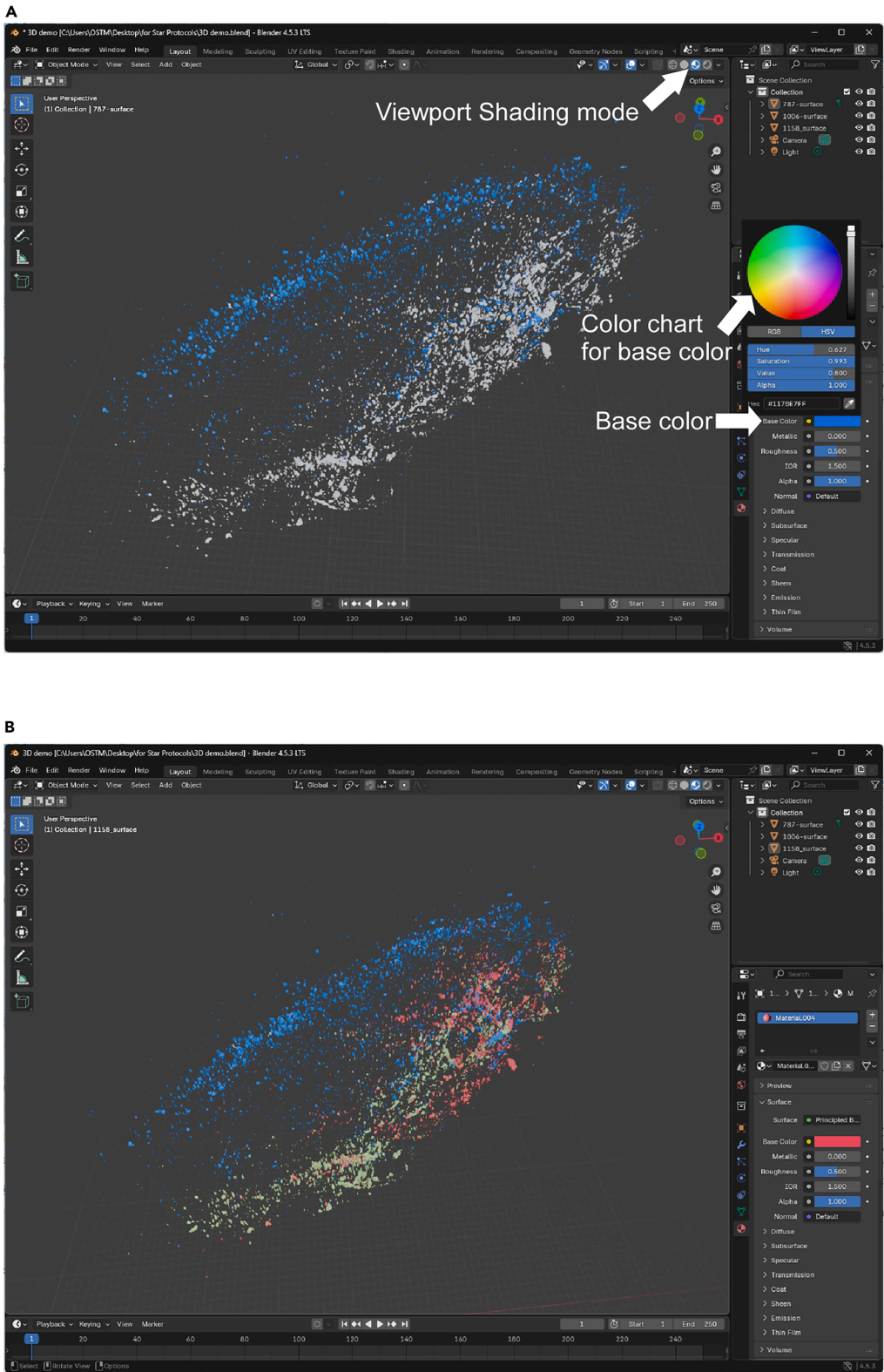


Figure 15. Blender interface for coloring

(A) Pseudo color is applied to the 3D data. The color chart for “Base Color” is shown. The “Viewport Shading” mode is used to visualize the 3D data in color.
(B) Three datasets displayed in different colors.

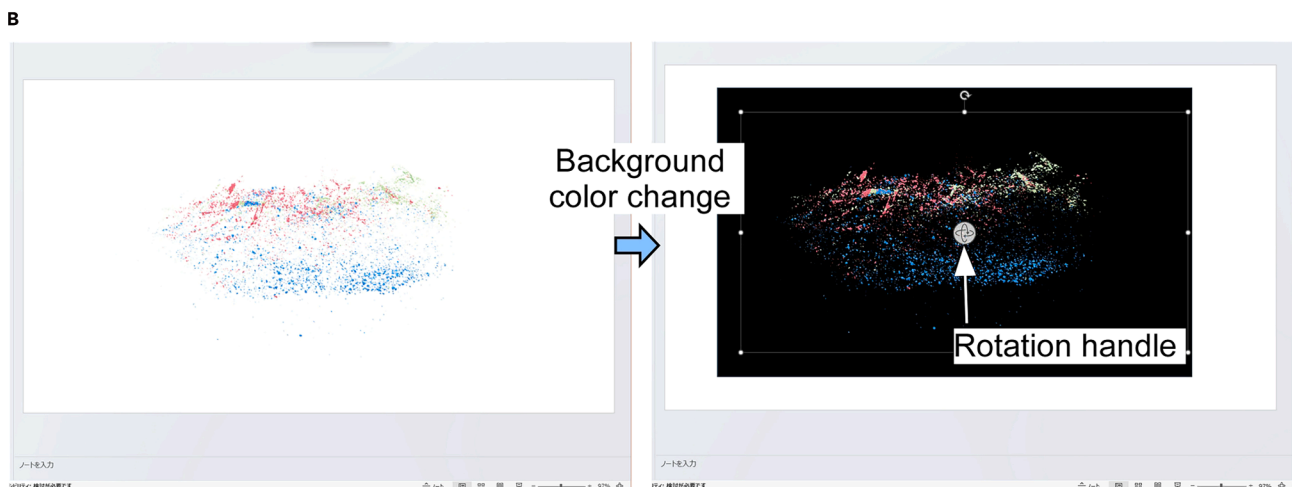
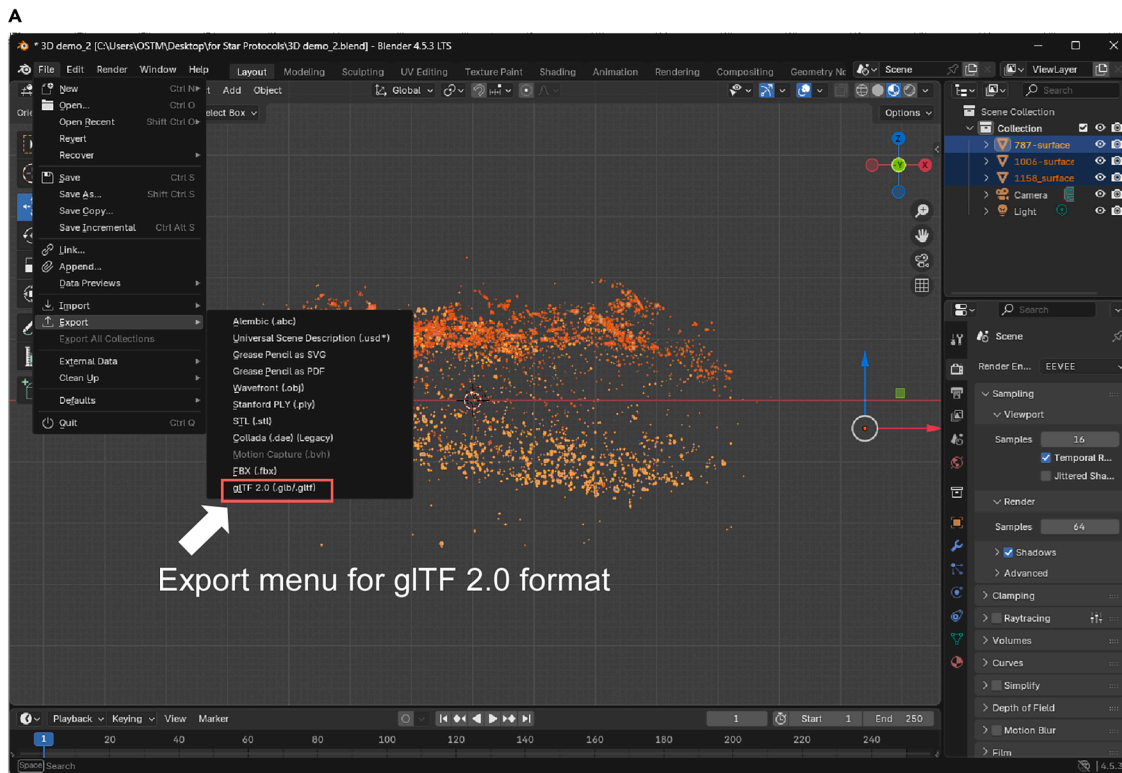


Figure 16. Exporting and inserting 3D data

(A) Exporting 3D data in Blender. The glTF 2.0 format is compatible with PowerPoint.

(B) Inserting 3D data into a PowerPoint presentation. Drag and drop the .glb file onto the slide and set the background to a dark color. The 3D object can be rotated by dragging the rotation handle.

AUTHOR CONTRIBUTIONS

T.O., A.S., and H.S. conceived the protocol with support from K.S., T. Yamamoto, and T. Yamagaki. Y.S. generated and provided the transgenic *Ciona robusta* line used in this protocol. T.O. wrote the original draft. All authors reviewed and edited the manuscript.

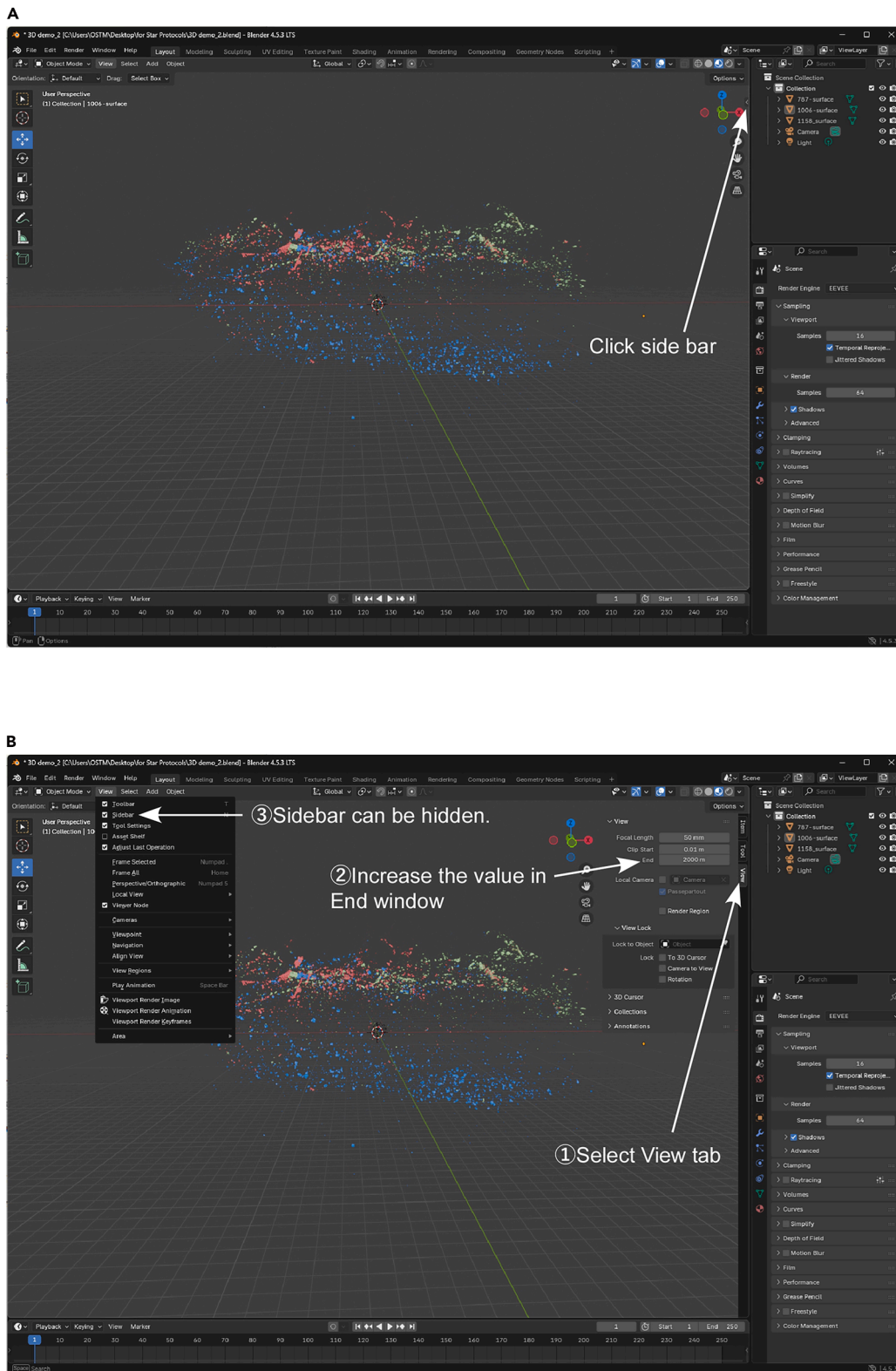


Figure 17. Trouble shooting 4

(A) The sidebar button is indicated by a small arrow. Side bar will appear when clicking the small arrow.
(B) Sidebar view. Three tabs (Item, Tool, and View) are displayed. The End parameter is set to 2000.

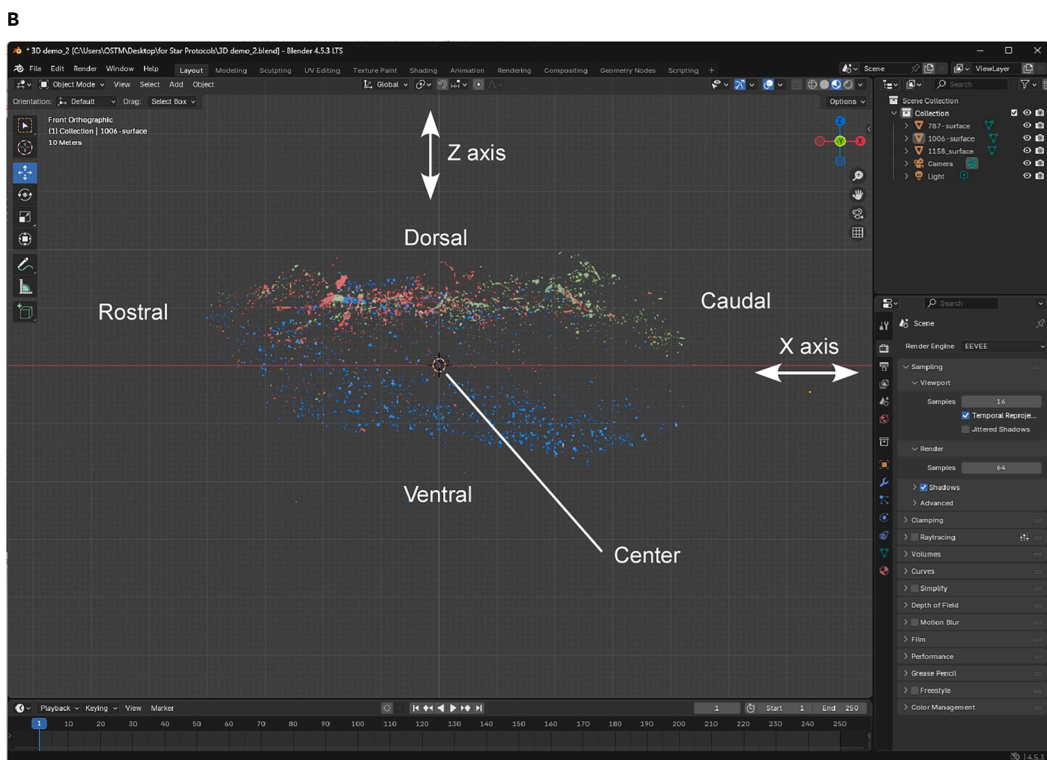
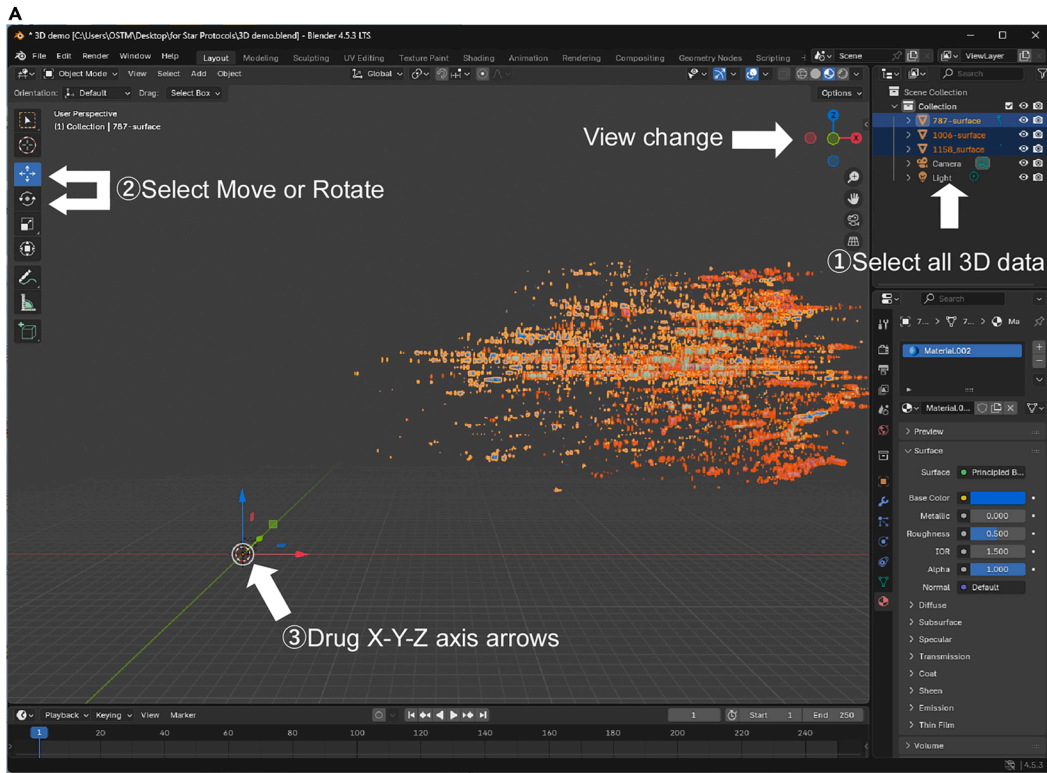


Figure 18. Troubleshooting 5

(A) The 3D datasets are listed in the Scene Collection window in the upper-right corner of the window. The “View change” button appears in the upper-right area of the viewport window. The “Move” or “Rotate” tools are located in the toolbar on the left. The X-, Y-, and Z-axis arrows are used to translate the 3D data.

(B) The 3D data aligned along with X-, Y-, and Z-axes. The anatomical directions of the *Ciona* cerebral ganglion (rostral, caudal, dorsal, and ventral) are indicated.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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