STOmics

MICROSCOPE ASSESSMENT GUIDELINE FOR StereoMap SOFTWARE

SoftwareVersion: StereoMap 4.0

Manual Version: C

STUM-PE001

REVISION HISTORY

Manual Version: A1
Software Version: V1.0 & V2.0
Date: May. 2023

Description:

- Initial release

Manual Version: B1
Software Version: V1.0
Date: Oct. 2023

Description:

- Added microscope evaluation procedures for Stereo-seq Transcriptomics Multi-immunofluorescence (mIF) solution and Stereo-seq Transcriptomics H&E solution.
- Added instructions for microscope hardware evaluation.
- Eliminated the evaluation requirements for blank Stereo-seq Microscope Assessment Chip T.
- - Modified compatible image formats (only compatible with TIFF/TIF formats).

Manual Version: C
Software Version: V4.0
Date: Jun. 2024

Description:

- Added microscope evaluation process that is compatible with Stereo-seq Transcriptomics FFPE product solution workflow.
- Added validated microscope models and their corresponding input file types.

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

TABLE OF CONTENTS

CRITICAL STEPS: Pay extra attention for these steps to avoid experimental setbacks or problematic results.

NOTE: Additional operation tips and guidance.

CAUTION: Proceed with extra care; improper handling or carelessness may cause experimental failure or accidents.

CHAPTER 1
INTRODUCTION

STOmics Stereo-seq Solution quantifies RNA of tissue sections followed by mapping and visualizing the transcriptomic data on an anatomical image. Typically, a high-magnification microscope is employed to capture high-resolution cellular images of tissues. Hence, the tissue image quality determines the success of downstream analysis and spatial clustering of gene expression data. This guidebook provides hardware recommendations, image acquisition and evaluation guidelines, as well as microscope compatibility test procedures. Additionally, an STOmics-developed image evaluation and processing software – StereoMap is also introduced in this guidebook.

1.1. Microscope Assessment Channels

a. Stereo-seq Transcriptomics application(ssDNA-stained nuclei image) -> FITC Channel

b. Stereo-seq Transcriptomics with mIF co-detection application (DAPI-stained nuclei image & mIF images) -> DAPI Channel

c. Stereo-seq Transcriptomics H&E application (H&E-stained image) -> Brightfield-Epi illumination

1.2. Assessment Software and Criteria

All microscope images are evaluated for image quality via the "Tools > ImageQC" module in StereoMap software, including the scoring evaluation of Trackline clarity and image clarity. All evaluations are based on the standard of Trackline QC score. For detailed scoring rules, please refer to **Chapter 4.2**, **Section ImageQC Scoring Instructions**. The image clarity score is for reference only and is not used as a pass/fail standard for image QC.

1.3. Assessment Duration

About half a day.

CHAPTER 2
MICROSCOPE HARDWARE EVALUATION

The microscope hardware evaluation mainly evaluates whether the microscope hardware parameters meet the Stereo-seq experiment requirements. The microscope hardware evaluation contents are as follows:

It is recommended to make an appointment in advance with your manufacturer's microscope engineer to accompany the evaluation on site.

Table 1 Overview of fluorescent imaging system requirements

Index/ Parameter	Description
XY stage travel distance of microscope	At least 25*75 mm
Imaging/Evaluation subject	Stereo-seq Microscope Assessment Chip Slide (Cat. No.: 201CT113)
Focusing approach	Pre-focus map and/or real-time autofocus
Objective lens	10X (NA ≥ 0.3)
Fluorescent channel	· DAPI (Excitation 358nm, Emission 461nm) · FITC filter cube (Excitation 480/40nm, Emission 525/50nm) · TRITC filter cube (Excitation 545/25 nm, Emission 605/70 nm) · CY5 filter cube (Excitation 620/50 nm, Emission 690/50 nm)
Camera resolution	Camera resolution selection depends on the objective lens. Please consult your microscope supplier for specific details
Image bit depth	· Grayscale image: 8bit/16bit (fluorescent image) · Color image: 3*8bit/3*16bit (H&E or other brightfield image)
Background balance	Adjustable background balancing function
Distortion correction	Adjustable distortion correction function
Overlap ratio	Adjustable, set it to 10%
Image format	Capable of viewing or exporting stitched images or Field-of-View (FOV) original images in TIFF format, 8/16 bit
PC requirement	Windows 10 x 64 system, 16G memory or beyond

Table 2 Overview of reflected light (epi-illumination) imaging system requirements

Index/ Parameter	Description
XY stage travel distance of microscope	At least 25*75mm
Imaging/Evaluation subject	Stereo-seq Microscope Assessment Chip Slide (Cat. No.: 201CT113)
Focusing approach	Pre-focus map and/or real-time autofocus
Fluorescent channel	DAPI, FITC, TRITC, CY5
Objective lens	10X (NA ≥ 0.3)
Camera resolution	Camera resolution selection depends on the objective lens. Please consult your microscope supplier for specific details
Image bit depth	Grayscale image: 8bit/16bit (fluorescent image)
Background balance	Adjustable background balancing function
Distortion correction	Adjustable distortion correction function
White balance	Adjustable distortion correction function
Overlap ratio	Adjustable, no less than 5%
File format	Capable of viewing or exporting stitched images or Field-of-View (FOV) original images in TIFF format, 8/16 bit
Pixel size	Maximum pixel size ≤ 5 μm/pixel
PC requirement	Windows 10 x 64 system, 16G memory or beyond

When outputting TIFF images from the microscope's software, please ensure that the image size and bit depth are consistent with the original format (such as CZI), as manual conversion of bit depth may lead to differences in QC results.

If the microscope hardware does not meet the above conditions, please switch to a different microscope and re-evaluate. The validated microscopes for Stereo-seq experiments are listed in **Table 2 (Chapter 2.1)**.

2.1. Imaging System Requirements

In general, an imaging system being used should satisfy these requirements:

1. Be able to perform tile scanning to image a minimal area of 10mm x 10mm (standard Stereo-seq Chip size).

This is required for the standard Stereo-seq Chip (1cm * 1cm). If large-sized Stereo-seq Chips are used for later transcriptomics experiments, please confirm the microscope scanning range in advance.

2. Have image stitching function either via the microscope's software (automatic stitching) or STOmics image processing software, StereoMap(manually assisted stitching, for FOV images only).
3. Have an image resolution ≥ 1800 pixels (height) and ≥ 2000 pixels (width).
4. Be able to view and save stitched images in raw file format (e.g., czi in Zeiss) and export it into TIFF.
5. The computer connected to the microscope is capable of processing large images (> 5GB) and supports the installation of third-party image processing software such as StereoMap.
6. The microscope should also have **ALL** the properties and functions listed in Table 3 or Table 4.

Table 3 shows microscope models used in the development of STOmics protocols.

Table 3 Validated imaging systems

Brand	Model/ Specification	Output image formats
Leica	Leica DM6-M	Original files in TIFF format
Zeiss	Zeiss Axio Scan Z1 Zeiss Axio Scan 7	Original Files in CZI format
MGI	STOmics Microscope Go Optical	Original files in TIFF format

The input files required for image registration differ based on the microscope used. At present, interface adapters for raw image file formats have been specifically designed for the validated microscope models mentioned in Table 3. For other microscopes, only stitched large images in TIFF format are supported as input files.

If none of the above microscopes is available,

a. Ensure that the microscope used fulfills hardware requirements stated in **Table 1** or **Table 2 of Chapter 2**.
b. Perform a microscope compatibility assessment following instructions provided in **Chapter 2.2**.

2.2. Imaging Configurations

Recommended Imaging Configuration for ssDNA - stained nuclei images:

Recommended Fluorescence Configuration:

- Light source with a wavelength range of 380 - 680 nm
- Monochrome camera (≥ 8 bit)
- FITC filter cube (Excitation 480/30, Emission 525/50)
- TRITC filter cube (Excitation 545/25, Emission 605/70)
- Maximum pixel size of 5 μm
- Exposure time 1 milli sec - 2 sec

Recommended Imaging Configuration for DAPI - stained nuclei images (mIF applications):

Recommended Fluorescence Configuration:

- Light source with a wavelength range of 380 - 680 nm
- Monochrome camera (≥ 8 bit)
- DAPI filter cube (Excitation 375/28nm, Emission 460/50nm)
- FITC filter cube (Excitation 480/30nm, Emission 525/50nm)
- TRITC filter cube (Excitation 545/25nm, Emission 605/70nm)
- CY5 filter cube (Excitation 620/50nm, Emission 690/50nm)
- Maximum pixel size of 5 μm
- Exposure time 1 milli sec - 2 sec

Recommended Imaging Configuration for H&E-stained images:

- Reflected light (Epi-illumination)
- Color camera (3*8 bit)
- White balance
- Maximum pixel size of 5 μm
- Exposure time 0.1 milli sec - 100 milli sec

CHAPTER 3
MICROSCOPE IMAGING GUIDELINES

3.1. Pre-assessment Preparation

Materials

- Stereo-seq Microscope Assessment Chip Slide (STOmics, Cat. No. 201CT113)
- One OCT/FFPE tissue block
- Microscope photograph image - Table 2 for input file formats.
- Experimental Procedure Manual - Table 4 for more details
- Software Operation Manual: StereoMap User Manual

Materials required for the experimental procedures differ based on the specific experimental workflows. For more details, please refer to the **STOmics Document List**.

Table 4 Overview of user manuals corresponding to different evaluation channels

Evaluation Channels	OCT embedded fresh frozen (FF) tissue block	Formalin-fixed and paraffin-embedded (FFPE) tissue block
FITC Channel	Stereo-seq Transcriptomics Set for Chip-on-a-slide User Manual	Stereo-seq Transcriptomics Set for FFPE User Manual
DAPI Channel	1. Stereo-seq Transcriptomics Set for mIF User Manual 2. Stereo-CITE Proteo-Transcriptomics Set User Manual	Stereo-seq Transcriptomics Set for FFPE User Manual
Epi-illumination	Stereo-seq Transcriptomics Set H&E User Manual	Stereo-seq Transcriptomics Set for FFPE User Manual

3.2. Microscope Assessment Procedure

This section applies to the microscope evaluation process of various product solutions based on STOmics Stereo-seq technology. Please select the appropriate evaluation procedures according to the project needs.

Please contact and make an appointment with a microscope engineer of the microscope brand you are using before the evaluation. Some microscope image parameters (such as FOV height, FOV width, and image scale) will be used during the evaluation process, and obtaining such information may require the assistance of the microscope engineer. After imaging the Stereo-seq Microscope Assessment Chip Slide and passing the QC, please confirm the image parameters and do not change them before the official Stereo-seq experiment.

3.2.1. Introduction to Experimental Procedure

The tissue preparation process for microscope evaluation is aligned with the various Stereo-seq experimental workflows, concluding with fluorescence or bright-field imaging. The specific workflow chosen depends on the embedding conditions of the tissue and channel requirements. Select the corresponding Stereo-seq User Manuals by referring to Table 4 in Chapter 3, Section 1 to access the latest version on the official **STOmics Website**.

Figure 1. Workflow for Microscopic Evaluation for Stereo-seq Experiments

3.2.2. Precautions for Experimental Operations

Stereo-seq Slide Cassette Assemble

Please refer to the Stereo-seq Chip Slide Assemble User Manual. For a demonstration video of Stereo-seq Slide Cassette assembling and removal, please refer to the link or by scanning the QR code: **https://en.stomics.tech/resources/videos/list.html**

Tissue Sectioning Instructions

Select the tissue sectioning method based on the tissue block type. Stereo-seq provides solutions for both frozen tissue blocks and paraffin blocks. Frozen sections are 10 μm thick, while paraffin sections are 5 μm thick for regular tissues and 4 μm thick for high-fat content tissues (e.g., breast tissue).

Tissue Mounted Chip Imaging Guidelines

1. Make sure to keep the chip aligned with the edge of the imaging stage and do not tilt the chip.
2. Adjust the imaging parameters to ensure that the exposure of the tissue area is well-balanced and the image, as well as the tracklines, is clear. If there is any problem, please contact the microscope engineer to troubleshoot within time.
3. It is recommended to use 'manual focus' for imaging. Select the modeling points first at the four corners of the chip and then select a few points within and outside of the tissue by focusing on them respectively, to obtain clear tracklines and tissue staining images at the same time. Please use 'autofocus' mode with caution. The autofocus might be tricky to adjust and fail to focus on both tracklines and the tissue, which might result in Image QC failure.
4. During manual focus imaging, first select the appropriate focal plane, exposure, and lighting parameters to identify the tissue contour clearly. Then, adjust these parameters in the blank areas to identify the tracklines clearly. Avoid setting lighting parameters too low or too high, as this can impact the image quality.
5. If there are regions (more than 4 FOVs) that have not been adequately infiltrated with mounting medium or glycerol, additional modeling points should be added to these regions.
6. Do not touch the surrounding surface during microscope scanning. Avoid introducing any vibration sources near the microscope, such as moving and walking.
7. Ensure the duration is within 30 minutes between tissue mounting to imaging. Do not leave your chip in the same position for a long time to avoid fluorescence signal quenching. Turn off the laser when the chip is not being imaged to prevent prolonged exposure, which may lead to local fluorescence quenching.
8. Compressed or scaled images will affect the QC and stitching results. Please ensure that the TIFF images exported by the microscope are not compressed or scaled to avoid image distortion affecting the downstream analysis.
9. After imaging, please use StereoMap software to QC the image immediately. If QC does not pass, please try to image it again.

CHAPTER 4
MICROSCOPE IMAGE EVALUATION

In this step, the obtained tissue images are input into the StereoMap software, where the **"Tools"** module is utilized for evaluation. The final assessment criterion relies on achieving a passing score for image trackline clarity evaluation. If Cellbin analysis is required, the image clarity score also needs to be taken into consideration.

4.1. Imaging Quality requirements

In general, a qualified image should fulfil the following requirements:

a. High image resolution: ≥ 1800 pixels (height) and ≥2000 pixels (width)

When exported an image file into TIFF format, ensure the file is not compressed to avoid resolution loss.

b. The imaged tissue contour and nuclei should be clear and within focus

c. Tracklines on the chip should also be clear and within focus
   1. To have both the imaged tissue and tracklines within focus, select multiple focal points when performing imaging.
   2. Set 3 focal points within the tissue and 3 other focal points in the background.
   3. Adjustment of contrast and brightness might be required after imaging, to have the tracklines clearly visible.

d. No stitching errors

e. Avoid photobleaching

f. Tissue area should not exceed 80% of the chip area

Figure shown here as an example of a high-quality mouse brain fluorescent image that has passed QC:

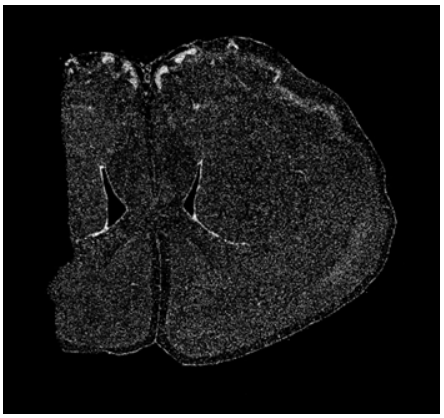


Figure 2

All the factors c. to f. will affect the image trackline QC score in Image Quality Control evaluation, which has a scale from 0 to 100. **A minimal score of 60 is required to pass the evaluation, the higher the score, the more accurate the image registration will be, which has a direct impact on data clustering.**

Please ensure that both the tissue and tracklines are in focus during imaging. If the microscope does not have automatic focusing capability or is not suitable, it is recommended to use manual focusing mode. It is typically necessary to set three focal points inside and outside the tissue to ensure comprehensive coverage. After imaging, adjust the contrast and brightness appropriately to clearly view the tracklines. During imaging, adjust the exposure parameters based on the clarity of the tissue/cells. Refer to the imaging guidelines for specific operations, and avoid overexposing tissues just to see track lines clearly. After imaging, rely on passing QC results or manually adjust brightness and contrast to view track lines as needed.

Imaging factors that will influence cell segmentation and cellbin results:

1. Capture images which cell boundaries can be clearly identified with naked eyes.
2. Image clarity score should be above 80.
3. Image (nuclei-stained image) MUST pass QC.
4. Make sure that the stitching error is ≤ 5 pixel (Contact and make an appointment with the manufacturer's microscope engineer for assistance if your image shows a stitching error > 5 pixel)
5. Tissue type and cell density will affect final cell segmentation in different aspects.

Cell segmentation will be greatly compromised if captured images fail to meet no. 1- 4, thus do not recommend to proceed with cellbin analysis.

4.2. StereoMap Image Assessment

StereoMap is an offline image processing software for Stereo-seq applications, primarily involving image quality assessment, manual adjustment modules (image calibration, tissue segmentation, and cell segmentation), and interactive visualization. Image quality evaluation mainly evaluates the clarity of the tracklines and overall image clarity of the microscopic image, ensuring suitability of the image for further downstream bioinformatics analysis.

4.2.1. Software Evaluation Workflow

1. Download and install the latest version of StereoMap software from the **STOmics website** and refer to the StereoMap user manual for installation instructions.
2. Execute Image quality control (ImageQC) module: **StereoMap->Tools-> ImageQC**. Different image files and formats are required to input into the software depending on the microscope models. Refer to the **StereoMap User Manual** for details.

4.2.2. ImageQC Scoring Instructions

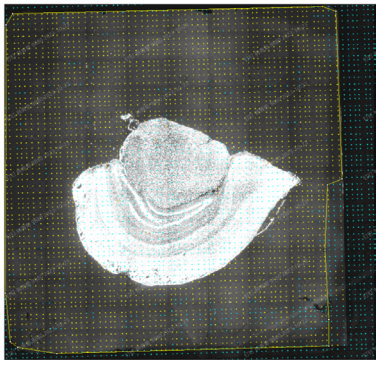
Trackline QC Score (applicable for ssDNA, DAPI, H&E, DAPI&mIF)

The ratio of derivable template points or area within 5 pixels to the template points or area possessed by the corresponding image of the whole chip is calculated respectively. The maximum of the two ratios is taken, and the maximum value is linearly mapped to a percentile score. If the ImageQC result of the trackline is 'Pass', meaning that the algorithm can successfully derive the global template.

- TemplateValidArea: The proportion of the surrounding area formed by the points of the derivation template with error less than 5 pixels when compared to the overall image area. As shown in this figure, the ratio of the pixel area surrounded by the yellow area to the pixel area of the entire chip = 460718882 pixels/604856088 pixels = 0.76

- TemplateRecall: The proportion of derived template points with an error of less than 5 pixels to the theoretical template points possessed by the corresponding image of the chip. As shown in this figure, the ratio of track points in the yellow doted matrix to the template points of the entire chip = 2636/4760 = 0.5538

Description	Trackline Score	ImageQC Results	Staining Type
TemplateValidArea, TemplateRecall) < 0.1	< 60	Fail	ssDNA/DAPI/ DAPI&mIF
(TemplateValidArea, TemplateRecall) ≥ 0.1	≥ 60	Pass	ssDNA/DAPI/ DAPI&mIF
(TemplateValidArea, TemplateRecall) = 1	= 100	Pass	ssDNA/DAPI/ DAPI&mIF
(TemplateValidArea, TemplateRecall) < 0.01	< 60	Fail	H&E
(TemplateValidArea, TemplateRecall) ≥ 0.01	≥ 60	Pass	H&E
(TemplateValidArea, TemplateRecall) = 1	= 100	Pass	H&E



Demonstration of Image trackline QC calculation process

Image clarity score (applicable for ssDNA & DAPI)

Image clarity score is calculated using a trained deep learning model which slices the input image, and divides the segmented image into five types: **1. good, 2. moderately blurry, 3. severely blurry, 4. slightly overexposed** and **5. heavily overexposed**, assigns different weighing points to each category, and finally calculates the clarity score of the entire image. The calculation formula is as follows:

$$ClarityScore = \frac{\sum_{n=1}^{5} w_n * c_n}{\sum_{n=1}^{5} c_n}$$

n represents the categories, **1 to 5** represents the 5 types of 'clarity', **w** represents the weighing points corresponding to the categories, and **c** represents the counts corresponding to the categories.

Clarity Types	Representative Number	Weighing Points	Staining Type
Good	1	1	ssDNA/DAPI/ DAPI&mIF
Moderately blurry	2	0.2	ssDNA/DAPI/ DAPI&mIF
Severely blurry	3	0	ssDNA/DAPI/ DAPI&mIF
Slightly overexposed	4	0.9	ssDNA/DAPI/ DAPI&mIF
Heavily overexposed	5	0	ssDNA/DAPI/ DAPI&mIF

This score shows the clarity of the tissue image and is used as a prediction of the final cell segmentation results. The higher the score, the better the quality of the tissue image and the higher the success rate of cell segmentation. The table below shows how the clarity score corresponds to the segmentation effect.

Clarity Score	Cell Segmentation Results
80 ~ 100	High success rate for automated cell segmentation
50 ~ 80	Moderate success rate for automated cell segmentation
0 ~ 50	Low success rate for automated cell segmentation

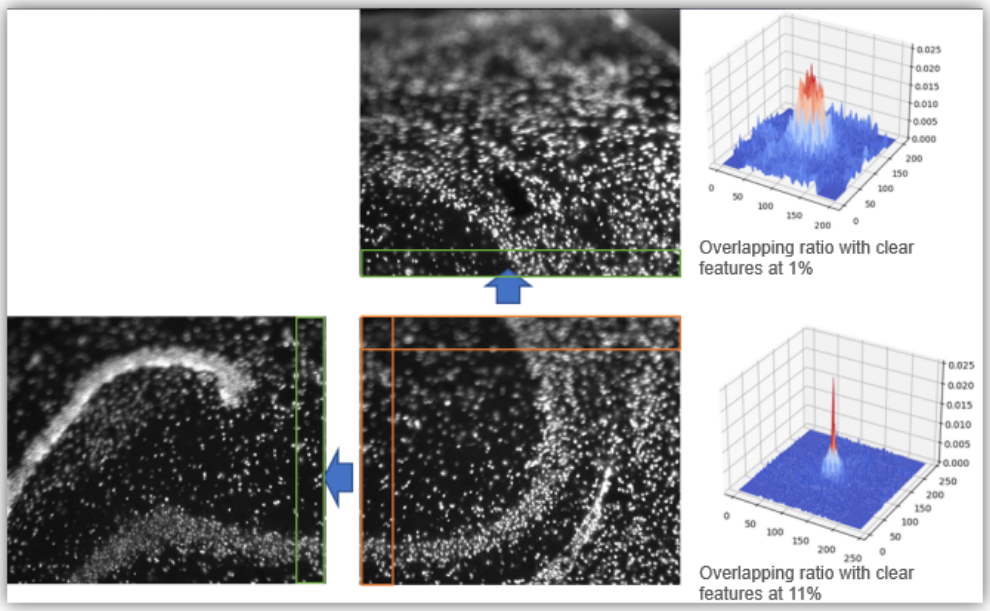
Automated cell segmentation is being done via Stereo-seq Analysis Workflow (SAW) pipelines.

The image clarity score does not affect the final QC assessment results and is only used as a reference to assess the feasibility of cell segmentation.

Microscope Stability Assessment Scoring Instructions (applicable for DAPI& mIF)

Microscope Stability Stitching Evaluation Score: Using an algorithm based on the similarity of overlapping regions, we divided FOVs into two types: FOVs with clear features in overlapping areas that can be detected by the algorithm and total FOVs. According to the ratio of FOV with clear features among the entire FOV that can be detected by the algorithm and the average offset score between each FOV and the adjacent FOV, the stitching score of each FOV is eventually calculated. This score is for evaluation of the stitching stability of the microscope, mainly for DAPI & mIF images, and for predicting image registration results. The higher the score, the higher the registration accuracy.
A good standard is that the boundaries of overlapping area features can be discerned by the naked eye. The definitions and calculation formulas of each index are as follows:

- **FOV with clear features in detected overlapping areas:** The number of FOVs that the algorithm can detect, represented in "a".
- **Total FOV:** The total number of FOV of the entire chip, represented in "b".
- **FOV offset score with clear features in the overlapping area:** Offset score calculated from the offset of each FOV and its adjacent FOV, represented in "c".
- **Average offset score:** The ratio of FOV offset score to the detected FOV numbers, represented in "d".



Example of overlapping area with clear features. The features of the overlapping area are clear when the ratio is greater than 5%.

Image Calibration Assessment Scoring Instructions (applicable for DAPI& mIF)

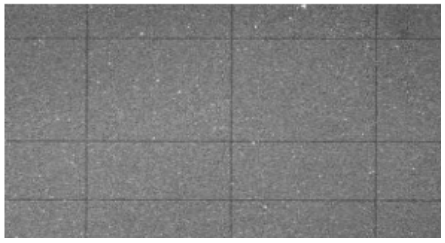
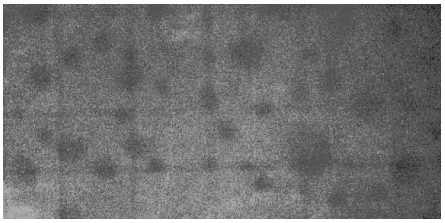
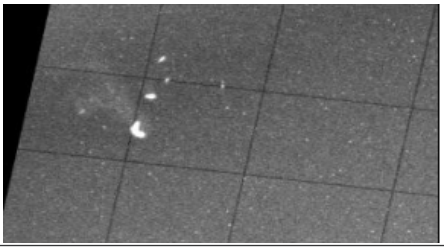
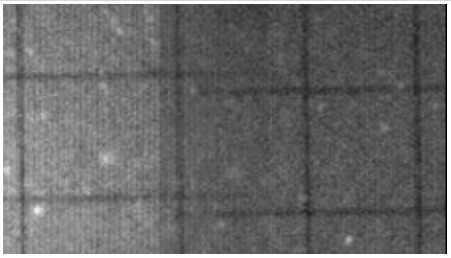
Image calibration assessment: It utilized a similarity calibration algorithm to evaluate the offset between DAPI and its corresponding IF images. The passing criteria are when the maximum offset does not exceed 20 pixels and the image similarity is greater than 1%. This score mainly evaluates the coincidence degree of DAPI and IF images, which predicts the image registration results.

Calibration evaluation is only for single-channel images, and the default DAPI and IF offsets for multi-channel images are 0.

Please refer to the Help Document section within the software for detailed instructions on software usage.

4.3. Image Examples

Examples below are images of good and bad qualities for comparison.

Tracklines		
Qualified image		
Unqualified image	Out-of-focus tracklines	
	Tracklines tilted	
	Stitching errors (Tracklines) [1]	

Tissue clarity

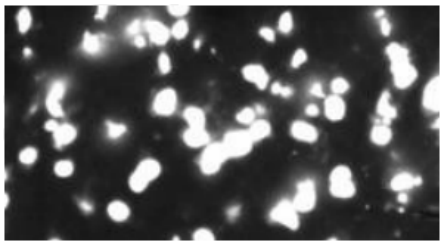
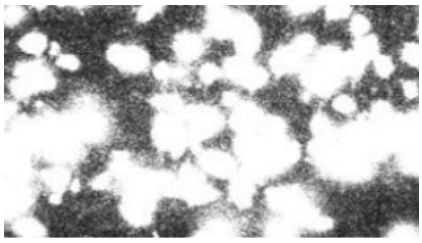
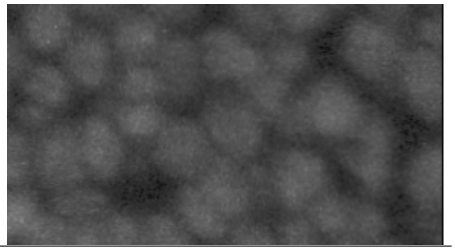
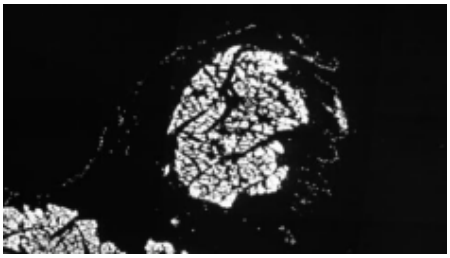
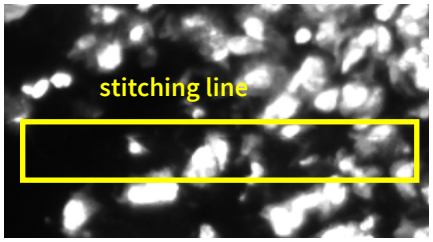
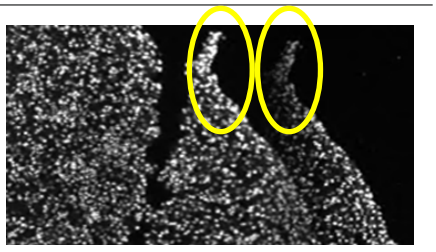
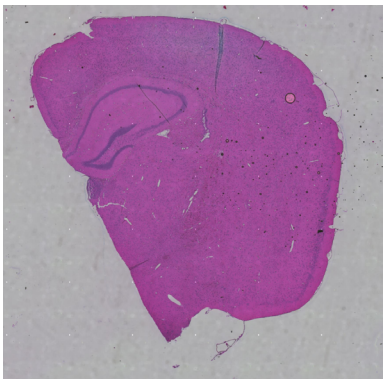
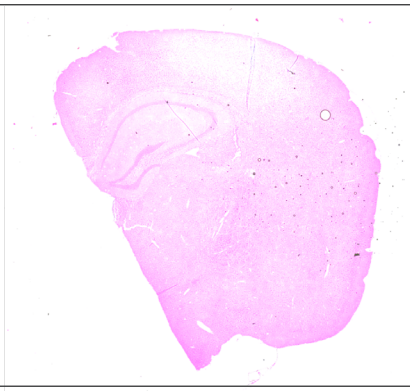
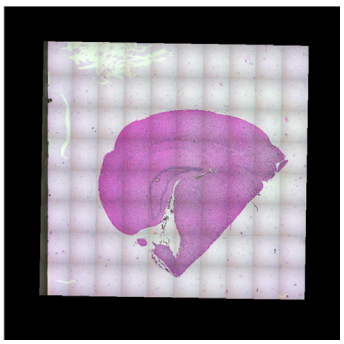
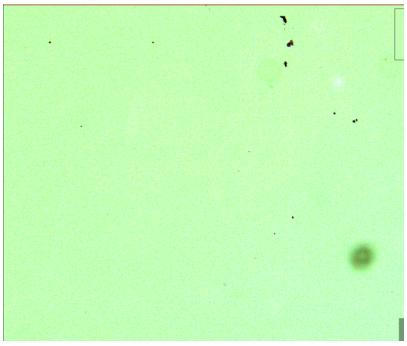
Qualified image		
Unqualified image	Overexposure	
	Out-of-focus tissue image	
	Indistinct tissue boundaries	

Image stitching

Qualified image		
Unqualified image	Stitching errors (Tissue image) [1]	

Epi-Illumination for H&E

Qualified image		
Unqualified image	Epi-Illumination overexposure	
	Epi-Illumination stitching artifacts[2]	
	Unadjusted white balance	

1. To solve stitching errors, please perform a microscope stitching calibration.

2. The appearance of distinct stitching lines is usually due to a strong brightness contrast among different tile images (FOV). Hardware repair/recalibration might be required, please contact the manufacturer's microscope engineer.