STOmics

Stereo-seq IMAGING REQUIREMENTS AND GUIDELINES

Manual Version: A

STUM-PE002

REVISION HISTORY

Manual Version:	A	**Description**:
Date:	Aug. 2025	· Initial release · Added the corresponding relationship between objective lens parameters and image resolution. · Updated the overview table of imaging system requirements. · Removed the list of microscopes tested for Stereo-seq experiments. · Added information on image formats and acquisition software versions supported by StereoMap. · Removed the table of biochemical experiment operation instructions corresponding to different evaluation channels. · Added a table of Stereo-seq imaging scenarios and corresponding biochemical experiment operation instructions. · Updated the operation precautions. · Refined scoring criteria for Tracklines. · Removed the description of image clarity scoring. · Removed the description of microscope stability assessment scoring. · Updated the calibration assessment instructions. · Expanded image examples and abnormal cases

Note: Please download the latest version of the manual and use it with the corresponding Stereo-seq Transcriptomics kit.

TABLE OF CONTENTS

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

CHAPTER 1
INTRODUCTION

Introduction

Stereo-seq technology is characterized by its ultra-large field of view (FOV) and subcellular resolution, allowing users to analyze complete tissue sections at the single-cell scale. Under normal circumstances, we use a high-magnification microscope to acquire high-resolution images of tissue cells. In subsequent analyses, we register gene expression data to these high-resolution tissue images and jointly conduct data mining and analysis at both the tissue and single-cell levels. This manual provides recommendations for microscope imaging systems, general imaging guidelines, and preliminary image assessment and analysis, aiming to guide users in evaluating the hardware parameters and imaging quality of the microscope to assist in confirming whether it can be used for the imaging phase of Stereo-seq experiments. In addition, this manual also introduces STOmic-developed StereoMap, the image assessment and processing software, as well as examples of images suitable for automated image analysis using Stereo-seq Analysis Workflow (SAW).

Applicability

This document, Stereo-seq Imaging Requirements and Guidelines (STUM-PE002), applies to multiple Stereo-seq products.

Please refer to the table below for the applicable Stereo-seq solutions and corresponding user manuals.

Product	Manual	Manual's No. (Click to view)
Stereo-seq Transcriptomics Solution	STUM-TT001 Stereo-seq Transcriptomics Set V1.3 for Chip-on-a-slide User Manual	View PDF
	STUM-PR002 Stereo-seq Permeabilization Set V1.1 for Chip-on-a-slide User Manual	View PDF
Stereo-seq Transcriptomics mIF Solution	STUM-TT003 Stereo-seq Transcriptomics Set V1.3 for mIF User Manual	View PDF
Stereo-seq OMNI for FFPE Solution	STUM-TT004 Stereo-seq OMNI Transcriptomics Set for FFPE User Manual	View PDF
Stereo-CITE Proteo-Transcriptomics Solution	STUM-TT005 Stereo-CITE Proteo-Transcriptomics Set V1.1 User Manual	View PDF
/	STUM-SP001 Sample Preparation Guide for Fresh Frozen Samples on Stereo-seq Chip Slides	View PDF
/	STUM-SP003 Sample Preparation Guide for FFPE Samples on Stereo-seq Chip Slides	View PDF

Overview of Evaluation Workflow

Based on actual applications and needs, select the corresponding STOmics technology, refer to the technical manual, If there are imaging requirements, it is recommended that you refer to this manual to conduct a preliminary assessment of the microscope's hardware parameters and capabilities. After the microscope passes the preliminary assessment, when imaging the Stereo-seq chip slide, first use the microscope's local software to determine if the imaging quality meets the requirements, and at the same time use the Tools module of the StereoMap software to assess the imaging conditions, such as tracklines. After the assessment of tracklines and other conditions passes, subsequent registration, segmentation, and other analyses can be carried out automatically. If the assessment does not pass, manual registration and other image processing analyses can also be performed using StereoMap, and then connected to SAW for subsequent analysis.

Individual results may vary due to differences among specific imaging systems and/or sample characteristics.

CHAPTER 2
IMAGING SYSTEM REQUIREMENTS

When visualizing gene expression data in conjunction with tissue images, it is essential to ensure that the resolution of the tissue images matches the resolution of the Stereo-seq technology (500 nm). Image resolution depends on three primary parameters:

- Objective lens resolution (determined by the numerical aperture (NA))
- Magnification of the objective lens
- Camera pixel size

The optimal image resolution varies according to the size of the target (tissue/cell/other markers), the contrast of staining characteristics, and the level of detail required. To achieve the best resolution, it is recommended that you follow the imaging settings recommended by the microscope manufacturer.

Stereo-seq technology can achieve subcellular resolution. Therefore, if there is a need for spatial single-cell resolution analysis, it is necessary not only to have clear imaging of tissue cells but also to ensure that the markers on the chip (tracklines) are clearly imaged. Based on this requirement, STOmics typically recommends that the image pixel size range be 0.3–0.75 μm.

Table 1 shows the parameters related to the objective lenses and the obtainable image conditions that meet the requirements of Stereo-seq experiments, as tested by STOmics imaging systems.

Images within the above resolution recommendation range are ideal, but if the downstream analysis of Stereo-seq data requires less detail, lower-resolution images can also be assisted by analysis with SAW and StereoMap software.

In addition to the example imaging systems provided in Table 1, any equivalent imaging system can be used as an alternative solution, depending on user needs and application scenarios.

Table 1 Examples of image resolution and file size obtained from objective parameters and target acquisition

Types of staining	Magnification of the objective lens /NA	Resolution of the objective lens (μm)	Image pixel size (μm)	Discernible features
ssDNA/DAPI	10X/0.3	1.1	0.345	Single cell/nucleus (5 μm); tracklines (1.5 μm)
IF	10X/0.3	1.1	0.345	Single cell/nucleus (5 μm); tracklines (1.5 μm)
H&E	10X/0.3	1.1	0.345	Single cell/nucleus (5 μm); tracklines (1.5 μm)

Types of staining	Bit depth of the image storage	File size (1*1 cm^2 Region)	Compatible file formats
ssDNA/DAPI	8 bit	1-2 GB (Single channel)	TIFF
IF	N*8 bit	N-2N GB (Single channel, N<=6)	TIFF
H&E	3*8 bit	3-4 GB	TIFF

Note: The above parameters are provided as an example based on the microscope parameters tested in STOmics. The camera pixel size is 3.45 μm.

For imaging scenes in STOmics experiments, it is recommended that you use an air objective lens with a magnification of 10x and a numerical aperture (NA) of 0.3 for image acquisition. If using higher magnification objectives (20x or higher) or higher NA (1.0 or higher), consider the following:

- The depth of field (DOF) will decrease. DOF refers to the thickness of the sample within which the focus appears in a plane of the image. As the NA of the objective lens increases, the DOF decreases. This can lead to increased difficulty in focusing on samples with a certain thickness, which in turn results in blurry images.
- The field of view (FOV) will decrease. To capture an image containing the entire tissue section, it will be necessary to acquire and stitch together more fields of view. This increases the possibility of stitching errors, which can result in misalignment of corresponding gene expression data.
- The generated images will be very large, with file sizes typically exceeding 3 GB and potentially reaching 100 GB (for large chips). Processing these images requires a computer with sufficient memory resources and specialized software to upload, visualize, and export the images as BigTIFF files. The processing time for handling large files can be lengthy; processing time is measured in minutes.

Note that, currently, the software provided with STOmics is only compatible with images generated using a 10x objective lens. If automated analysis is required for images generated using other magnification objectives, the images should be converted to the corresponding image resolution under a 10x objective lens.

For example, if the original images are captured using a 40x objective lens, images should be resized to 1/16 of original dimensions prior to software.

The above provides a detailed description and recommendations for the key parameters of the imaging system. However, in practice, whether the final imaging results meet the requirements depends on the overall design of the imaging system. The following are the general requirements for an imaging system in most cases.

Table 2 Overview of imaging system requirements

Imaging System and Performance Metrics	
Metrics/Parameters	**Description**
Motorized Stage	Motorized control is supported in the X, Y, and Z-axis directions.
Microscope XY axis travel range (stroke)	The scanning area range covers at least a 10 mm x 10 mm area. The example given is based on the STOmics 1 cm x 1 cm chip size. If a larger chip size is required, confirm the scanning range of the microscope in advance.
Capture Target	Stereo-seq Chip Slide
Focus Mode Evaluation	Pre-focusing map or real-time autofocus (photoelectric autofocus or map-based selection)

Imaging System and Performance Metrics	
Metrics/Parameters	**Description**
Observation Mode	Epifluorescence, Epipolar Brightfield
Scanning Functionality	Automated scanning, automatically stitching the captured fields of view into a large image
Fluorescence Channels	DAPI, FITC, TRITC, CY5
Objective Lens	4X, 10X (NA>=0.3, Air objective lens, achromatic or semi-apochromatic lens for chromatic aberration correction)
Resolution	Consult the microscope supplier for information on matching the camera resolution to the theoretical resolution of the objective.
Camera and Image Bit Depth	Fluorescence: Monochrome camera, 8/16 bit Brightfield: Color camera, 3*8 bit/3*16 bit
Image Acquisition	• Calibrate the microscope and camera according to the manufacturer's instructions, including adjustment for motion stability, distortion correction, background balancing, and white balance. • Adjust imaging settings (light source power, camera gain, and exposure) to achieve bright and clear images.
Image Format	View and export stitched large images/FOV original images in 8/16 bit, TIFF format.
Software	Microscope scanning software, image viewing software, and image processing software (e.g., Scanner, StereoMap, QuPath, FIJI/ImageJ).
Computer System Requirements	Win10 x64 system, minimum 16 GB of RAM, with sufficient memory to handle large images.

Note: Any equivalent system with the listed functions can be used for imaging.

Note: The imaging systems listed above have specific requirements and recommendations. Consult the microscope manufacturer for reference and evaluation, and then ensure that calibration and adjustment are completed. If you have already taken photos with the microscope evaluation chip T, and the systems pass QC in StereoMap, confirm all parameter information. Do not make any changes without proper consideration before the actual experiment.

CHAPTER 3
MICROSCOPE IMAGING GUIDELINES

3.1. Preparation before Imaging

Materials

- Stereo-seq chip slide (Stereo-seq Microscope Assessment Chip Slide, Cat. No.: 201CT113)
- 1 OCT/FFPE tissue embedding block
- Microscope imaging system (specific requirements can be found in Chapter 2)
- Experiment operating instructions (see detailed instructions in Table 3)
- Software operating instructions (see detailed instructions in the StereoMap User Manual)

Select the required materials and experiment procedures based on the specific experiment. The instructions can be found in **STOmics Global Website -> Resources**.

Table 3 STOmics imaging scenarios and the corresponding user manual for each biochemical experiment

Imaging Mode	Image type	OCT tissue block (Follow SP001 for sample prep.)	FFPE tissue blocks (Follow SP003 for sample prep.)	Demand point	Application function points
Epi-FL (Epi-fluorescence)	ssDNA (single-stranded DNA, FITC channel)	Follow TT001 up to Section 3.6, then switch to PE002 3.2	Follow TT004 up to 3.4, then switch to PE002 3.2	Tracklines, Tissue, Cell	Cell-level Registration, Tissue and Cell Morphology Information
	DAPI (4',6-dia-midino-2-pheny-lindole)	Follow TT003 up to Section 3.7, then switch to PE002 3.2 Follow TT005 up to Section 3.7, then switch to PE002 3.2	Follow TT004 up to 3.4, then switch to PE002 3.2	Tracklines, Tissue, Cell	Cell-level Registration, Tissue and Cell Morphology Information
	TRITC (Tetrame-thyl-rhodami-neiso-thiocy-anate)	Follow PR002 up to Section 3.9, then switch to PR002 3.2	-	Tissue, Cell	Permeabili-zation Optimization Experiment, Tissue and Cell Morphology Information
	CY5 (Cyanine 5)	Follow TT003 up to Section 3.7, then switch to PE002 3.2	-	Tissue, Cell	Tissue and Cell Morphology Information

Imaging Mode	Image type	OCT tissue block (Follow SP001 for sample prep.)	FFPE tissue blocks (Follow SP003 for sample prep.)	Demand point	Application function points
Epi-BF (Epi-brightfield)	H&E (Hemato-xylin-Eosin)	Follow TT001 up to Section 3.6, then switch to PE002 3.2	Follow TT004 up to 3.3, then switch to PE002 3.2	Tracklines, Tissue, Cell	Cell-level Registration, Tissue and Cell Morphology Information

Please refer to Chapter 1, "Introduction – Applicability" for the user manual numbers of corresponding Stereo-seq Solutions.

3.2. Microscope Imaging Procedure

This chapter applies to the microscope imaging workflow corresponding to various product solutions based on Stereo-seq technology. Select the appropriate workflow based on your specific needs. Taking the Stereo-seq Chip T Slide as an example to introduce the entire imaging operation process.

3.2.1. Process Introduction

The tissue preparation process should be consistent with the experiment procedures. Choose appropriate Stereo-seq biochemical experiment user manual following Table 3 in Section 3.1, depending on the tissue block types and Imaging channels.

Obtain the latest version of the user manual from the STOmics Global website: https://en.stomics.tech/resources/documents/list.html.

Before evaluating the imaging channels, the experiment must be completed up to the fluorescence or brightfield imaging step. The diagram below outlines a general workflow applicable to all solutions. For detailed procedures under different channel conditions, please refer to Table 3 in Section 3.1.

Figure 1. Overview of the biochemical workflow prior to microscope image evaluation

3.2.2. Operation Precautions

Demonstration Video of Slide Cassette Assembly and Removal

For a demonstration video of Stereo-seq Slide Cassette assembly and removal, please refer to the link or scan the QR code: **https://en.stomics.tech/resources/videos**.

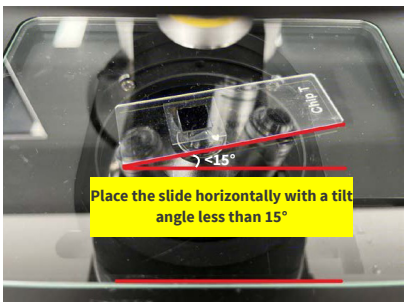
Tissue Sectioning Instructions

Select the tissue sectioning method based on the tissue block type. Stereo-seq provides solutions for both fresh frozen (FF) tissue blocks and formalin-fixed and paraffin-embedded (FFPE) blocks. For fresh frozen samples, the recommended section thickness is 10 μm, while FFPE samples typically require 5 μm sections, or 4 μm sections for high fat content tissues (e.g., breast tissue).

Imaging Precautions

a. **Chip Placement:** Gently place the chip on the stage, ensuring the chip back side with the serial number (SN) at the top and the QR code at the bottom. For chip-on-slide versions, orient the engraved label to the right-hand side; bare chips follow the same placement rule with the QR code on the back at the bottom.

Chip Placement Diagram (backview): Chip number on top, QR code at the bottom.

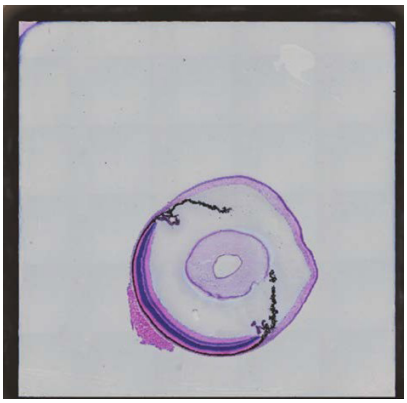


Chip Placement Diagram (top view): The engraved label is on the right.

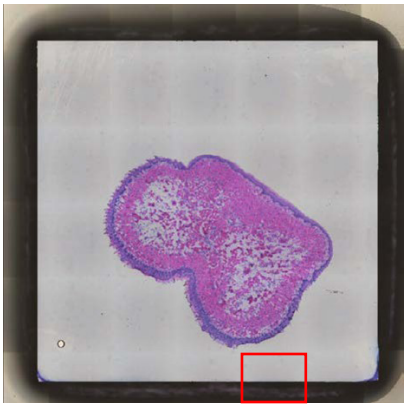
b. **Parallel Alignment:** The chip should be positioned as parallel as possible to the stage, with the edge horizontal angle maintained at less than 15° to minimize tilt errors during imaging.

c. **Imaging Consistency:** Under the requirements of a) and b), ensure that the imaging direction of the microscope is consistent with the actual orientation of the specimen.

d. **Imaging Scope:** During imaging, ensure the chip edges are fully captured, with all four corners and edges clearly visible. The distance between the chip edge and the image edge should be kept to no more than half the width of the FOV. As shown in the figures below, both are correct examples. In the left image, the chip edge is close to the image edge, while in the right image, the chip edge maintains approximately half the FOV distance from the image edge.



Acceptable Image Example (edge-aligned)



Acceptable Image Example (within half of the FOV)

e. **Focus and Exposure Adjustment:** Properly adjust focus and exposure parameters to ensure that both the tracklines and tissue regions are clearly imaged simultaneously. Note that there is a significant difference in focus and fluorescence brightness between the tracklines and tissue regions; refer to the user manual for imaging.

f. **Focus Mode Selection:** If manual focusing is used, modeling points should be selected at the four corners of the chip (non-tissue areas) and within the tissue to set the focus in order to obtain clear images of the tracklines and tissue. Use automatic focus mode with caution; the autofocus strategies of most microscopes cannot accurately focus on the tracklines, which can result in QC failure.

g. **Vibration Avoidance:** During microscope scanning, avoid touching the surface on which the microscope is placed, and ensure that there are no sources of vibration in the vicinity, including walking or other activities that could cause disturbances.

h. **Mounting Medium Coverage:** If there are large areas (exceeding the size of four FOVs) that are not fully covered by the mounting medium, additional modeling points should be added to these areas.

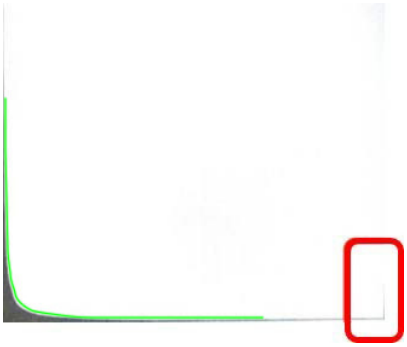
i. **Image Compression and Scaling:** Compressed or scaled images can affect the image QC and stitching results. To avoid image distortion that may affect the analysis results of subsequent processes, ensure that the TIFF images exported from the microscope are not compressed or scaled (under 10X conditions). The inspection method is visual confirmation.

j. **Image Quality Control (QC):** Immediately after taking photos, use the StereoMap software to perform image QC. If the QC fails, retake the photos promptly.

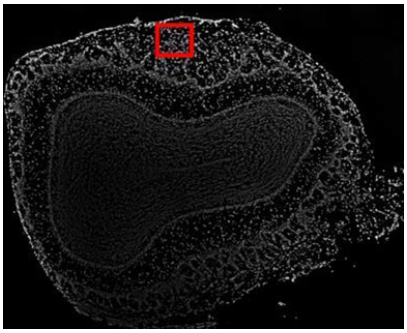
k. **Tissue Area and Trackline Area:** If the tissue area exceeds 80% of the chip area and the trackline area is insufficient, the likelihood of successful automatic registration via tracklines decreases. In such cases, manual registration should be employed.

The above are general precautions for the process. For specific imaging procedures according to different needs and applications, refer to the corresponding user manual (Table 3 in Section 3.1). For microscopes that require manual setting adjustments and verification, pay attention to the following:

1. When conducting single-cell level analysis, the image processing output must achieve a matching resolution. Taking the mouse brain (cell diameter ≈ 10 μm) as an example, if the stitching error exceeds half a cell, the analysis cannot achieve single-cell level resolution, it is usually caused by a large movement deviation of the stage. Contact the microscope manufacturer's engineer for calibration; you can also export FOV images and re-stitch them using third-party software (such as Fiji, ImageJ).
2. After stitching, the image may exhibit uneven brightness at the junction of each FOV (stitching marks). Under fluorescence imaging, possible causes for troubleshooting include weak sample fluorescence signals, strong autofluorescence of the sample itself, and poor flatness of the stage.
3. Apart from the imaging of permeabilization time optimization, the situation in **item 2**. can be improved by the microscope's background balancing function which can be performed in a blank area. However, it should be noted that the background area usually has tracklines, and tracklines should not be visible in the FOV (you can slightly defocus). Otherwise, double tracklines would appear in the final image, which will affect subsequent analysis. This is an important note for Stereo-seq imaging, if fixed template is available, it is recommended to use it for background balancing correction.
4. For brightfield imaging, it is important to select a blank area on the chip and perform white balance adjustment to adjust the color balance of the camera. If there is any problem, contact the microscope manufacturer’s engineer for calibration.
5. When multiple fluorescent stainings are required for the sample, it is necessary to verify whether there is any crosstalk between different fluorescence channels because crosstalk may affect the recognition of single cells. Contact the microscope manufacturer's engineer for assistance with calibration. It is necessary to confirm whether there is any offset between images captured by the different channels. If offset is detected, use third-party software (such as Fiji, ImageJ, etc.) for calibration.
6. During fluorescence imaging, it is important to avoid overexposure as much as possible. This can be judged by the image pixel statistics (such as histograms). The criterion for optimal exposure: the histogram shows a moderate curvature, and the curve does not touch the bottom left of coordinate axis. Besides, there is only a slight protrusion at the maximum value of the horizontal coordinate of the histogram (as highlighted in the red box in the figure below). For brightfield imaging, keep the brightness parameter at a moderate level, set the gain to the lowest, and adjust the exposure appropriately.



7. During fluorescence imaging, parameter adjustments are required during the manual focusing process. First, focus on the tracklines, and you can increase the gain and light intensity to ensure that tracklines are visible. After selecting the tracklines points, when focusing on the tissue, reduce the gain to the lowest and select a tissue area with high coverage and comparatively bright (the area within the red frame in the figure below), adjust the exposure time (not exceeding 500 ms) and light intensity (increase the light intensity if the 500 ms exposure does not meet the requirements) to ensure that this area is not overexposed. Finally, take the exposure parameters of the tissue area for imaging.



8. During fluorescence imaging, prolonged laser exposure can cause photobleaching. To ensure optimal imaging results, it is recommended that you complete imaging within 30 minutes upon sample mounting (reference: 1cm * 1cm chip).

3.3. Image Format And Size Requirements

SAW 8.0 and StereoMap 4.0, as well as higher versions, are compatible with TIFF (.tif, .tiff) image formats. It is recommended that you install the latest version of the StereoMap software before the imaging assessment. StereoMap software aims to support manual image processing operations such as trackline assessment, manual registration of tissue staining images, tissue region selection, and cell segmentation.

Images from individual fluorescence channels (e.g., mIF applications) can be opened and processed separately, with a maximum of six files per tissue section. It is necessary to ensure that each monochrome image is obtained at the same magnification, with the same bit depth, size, orientation, and file format. If imaging is done separately for each channel, ensure that the offsets between channels meet the analysis requirements.

When the acquisition software cannot save or convert images in one of the compatible format types, use third-party software, such as FIJI/ImageJ, for conversion.

Preferred Input and Alternative Options:

Stitched images are recommended as the primary input. If stitched images cannot be used due to stitching errors, acquisition tiles can be used as an alternative (StereoMap supports the input of acquisition tiles from certain microscopes; refer to the table below for specific supported information). Additionally, images stitched by third-party tools are also supported.

Table 4 Supported microscope original tile directories and formats in StereoMap

Image file formats	Microscope brands	Microscope acquisition software version information
Acquisition tiles in .tiff format	STOmics	Scanner Version 1.2.2
Acquisition tiles in .tif format	Motic	PA53 Scanner 1.0.0.14
Acquisition tiles in .tif format	Leica	LAS X 3.7.4.23463
Files in .czi format	ZEISS	ZEN Version 3.1.0.0000 ZEN Version 3.5.093.00009

Note: Due to an inability to synchronize updates to the microscope image file directory structure and file formats of each microscope brands in a timely manner, if there are any issues, promptly export the stitched large image for subsequent analysis.

CHAPTER 4
MICROSCOPE IMAGE EVALUATION

Based on different application requirements, conducting immediate assessments of the produced images can help users decide whether to retake the images. If better image quality cannot be achieved through optimization of the imaging process, it is also possible to make preliminary judgments about the image conditions to anticipate subsequent analysis plans. The application scenarios of Stereo-seq technology are diverse, and the image requirements vary accordingly. Therefore, manual image evaluation is essential for optimizing acquisition parameters to meet quality requirements or adapting analysis protocols for specific application needs.

To enhance the product experience, STOmics provides the StereoMap software to help users assess image quality. The most important aspect is the evaluation of the chip marker lines (tracklines), which is a rigid requirement for subsequent automated registration and will serve as one criterion for image quality control results.

4.1. Imaging Quality Requirements

In general, high-quality images typically meet the following characteristics:

1. The imaged tissue and cells are clear and within the focal range.
2. Tracklines on the chip are clearly imaged and distinct.
3. There are no stitching errors.
4. There is no photobleaching, the overall brightness of the tissue is even, and the chip area is clean and does not contain impurities.
5. The tissue coverage area does not exceed 80% of the total chip area, and the edges of the chip are within the image.

The following figure is an example of a high-quality image that has passed QC:

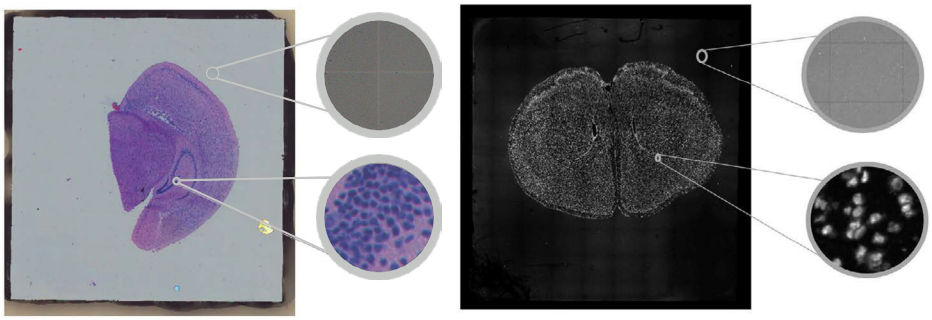


Figure 2. Example of high-quality images (tissue, cells, tracklines)

During the imaging process, refer to the imaging guide for capturing images. After the imaging is completed, use the microscope's local viewing software to review the images as a whole. First, ensure that the tissue and cells in the region of interest are clearly visible to the naked eye, then adjust the contrast parameters to verify that the tracklines are clearly visible and determine if there are stitching errors. If you wish to use the SAW software for automated CellBin analysis, make sure that the tracklines QC has passed and that the tissue and cells are clearly visible to the naked eye, with no significant stitching misalignment (all at the single-cell precision). If the QC fails or the assessment is not satisfactory, consider whether you need to retake the photos or use manual image processing methods.

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 registration, tissue segmentation, and cell segmentation), and interactive visualization. Image quality evaluation involves evaluating the clarity of the tracklines and overall image clarity of the microscope image for the purpose of determining how to use StereoMap and SAW for 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 the image quality control module: **StereoMap->Tools->ImageQC**. Refer to the **StereoMap User Manual** for details.

4.2.2. ImageQC Scoring Instructions

Tracklines QC Score (Compatible with ssDNA, DAPI, H&E, DAPI + mIF applications)

When using tracklines features for registration, the following key points must be considered:

1. At least five neighboring points, including at least one clear track cross, should be visible - this is an extreme case; under normal circumstances, at least 10 points should be achieved based on this principle.
2. To ensure the accuracy of the registration across the entire chip area, at least three external corners of the tissue's bounding rectangle must have the situation described in item #1 above).
3. The more accurately the track points can be detected the better, and they should be evenly distributed on the image with the situation described in item #1 above as the minimum unit.

Based on the above, the tracklines scoring rules are set as follows:

After detecting, filtering, and calculating the scale and rotation parameters based on track points, a track point template derived from the image track points is produced. The error between template track points and those detected by the QC inspection model is calculated (error <5 pixels). The following two metrics are then established:

TemplateValidArea: The proportion of the enclosed area formed by the number of derived template points within 5 pixels of error to the overall image area. As shown in Figure 3, the ratio of the pixel area enclosed by the yellow area to the total pixel area of the image: 460718882 pixels / 604856088 pixels = 0.76.

TemplateRecall: The proportion of the number of derived template points within 5 pixels of error to the theoretical number of template points that the corresponding image on the image should have. As shown in Figure 3, the ratio of the track points in the yellow dot matrix to the total number of template points on the image: 2636 / 4760 = 0.55.

Take the maximum of the two ratios and linearly map the maximum value to a percentage score. The tracklines QC status is successful, which means that the algorithm can correctly derive the template through the image track points, and the preliminary registration is successful.

Table 5 Tracklines scoring

Description	Tracklines score	Tracklines QC results	Staining type
(TemplateValidArea and TemplateRecall) <0.1	<60 score	Fail	ssDNA/DAPI
(TemplateValidArea or TemplateRecall) ≥0.1	≥60 score	Pass	ssDNA/DAPI
(TemplateValidArea or TemplateRecall) =1	=100 score	Pass	ssDNA/DAPI
(TemplateValidArea and TemplateRecall) <0.01	<60 score	Fail	H&E
(TemplateValidArea or TemplateRecall) ≥0.01	≥60 score	Pass	H&E
(TemplateValidArea or TemplateRecall) =1	=100 score	Pass	H&E

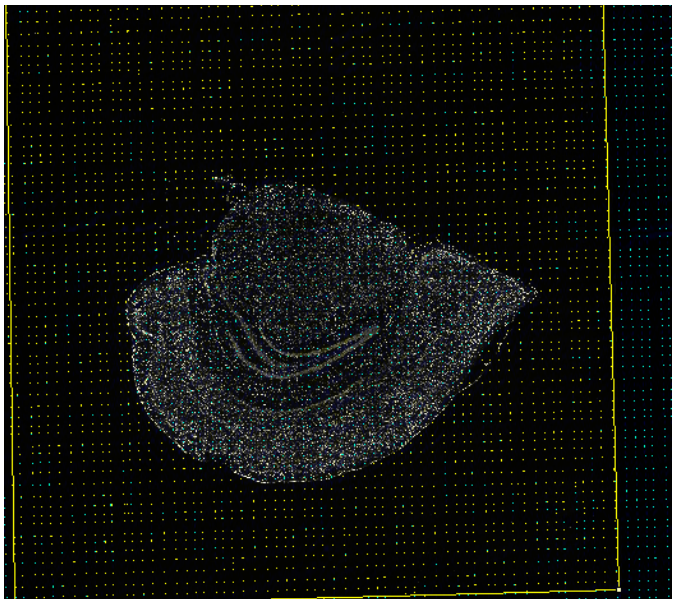


Figure 3. Tracklines scoring diagram

Calibration Assessment Instructions

During the process of multi-channel fluorescence staining imaging, if imaging with multiple single channels is applied, there may be offsets between different fluorescence images. The StereoMap software employs a similarity calibration algorithm to assist in assessing the overall offset of multi-channel fluorescence images. Because multi-channel images capture different information from the tissue, similarity cannot be guaranteed. This part is only used for offset assessment scoring and does not involve calibration operations, nor is it included in the QC content. When using StereoMap to review data results, manually confirm whether the fluorescent images meet the requirements for subsequent analysis. If they do not meet the requirements, use third-party tools for calibration before proceeding with further analysis using StereoMap and SAW.

Note: The calibration assessment applies only to the single-channel multi-imaging mode where images are captured separately. For multi-channel simultaneous imaging, the default offset is assumed to be 0.

4.3. Image Examples

The following imaging anomalies may result in low image resolution, failure to align microscope images to gene expression maps, failure to analyze images, and/or difficulty in interpreting data. Before starting formal experiments with Stereo-seq technology, make sure that the microscope meets the imaging requirements for the Stereo-seq experiment and achieves optimal imaging settings. The following examples are from representative tissue sections. Refer to the corresponding kit instruction documents for compatible species and tissue types.

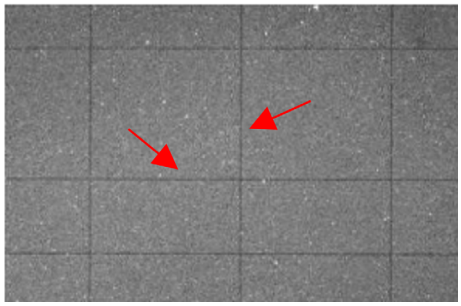
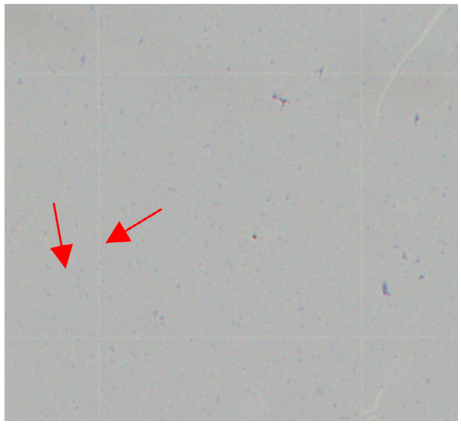
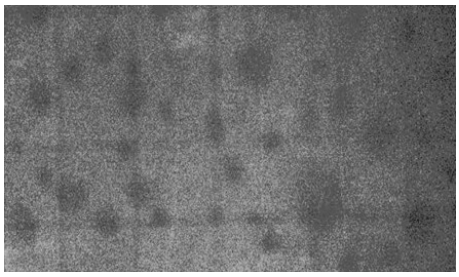
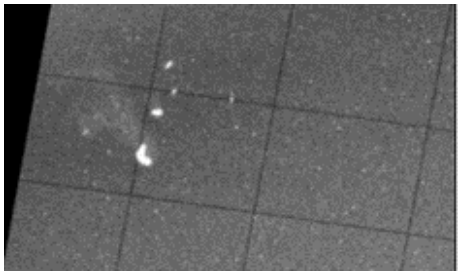
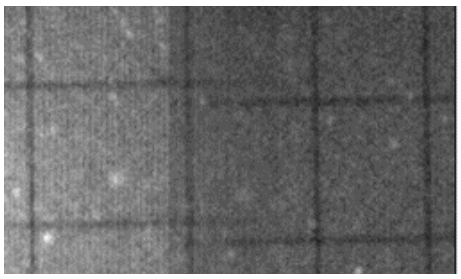
<table>
<tr><th colspan="2">Tracklines</th></tr>
<tr><th>Qualified image</th><th>Unqualified image</th></tr>
<tr><td> 
Fluorescent image
 
H&E</td><td> 
Out-of-focus tracklines
 
Tracklines tilted
 
Stitching errors
(tracklines)</td></tr>
<tr><td>The tracklines are mostly observable in non-tissue areas by adjusting the contrast. On fluorescence images, the tracklines appear as dark lines due to their lower brightness. In brightfield imaging, the tracklines are visualized as white lines.</td><td>Tracklines are straight lines arranged on the chip and are generally parallel to the edges of the chip. Out-of-focus imaging can lead to unsuccessful trackline detection. Tilting the chip can also reduce detection accuracy. If there is a stitching error, clear trackline displacement can be observed.</td></tr>
</table>

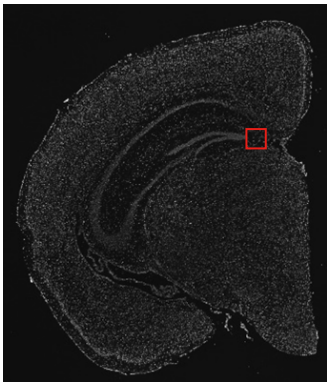
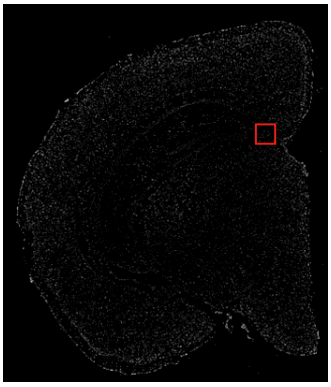
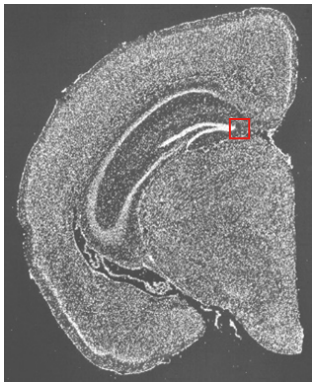
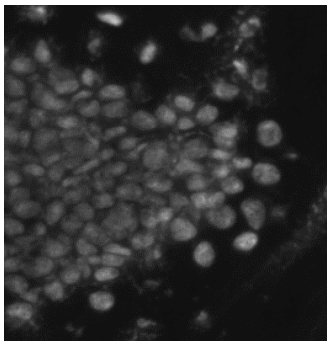
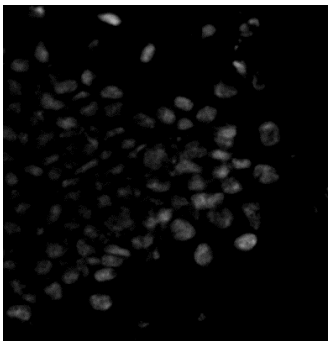
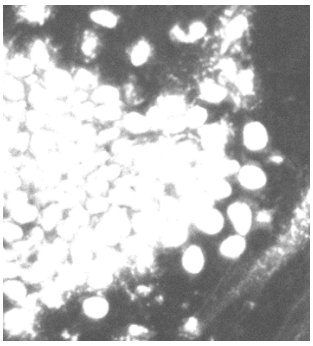
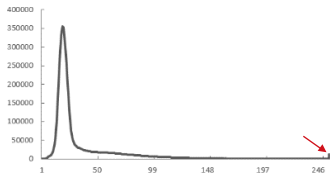
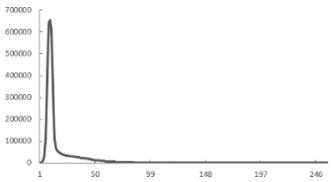
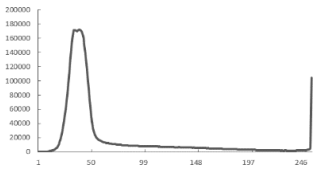
Image exposure (fluorescence)		
Qualified image	**Unqualified image**	
		
		
		
Proper exposure can provide good brightness and contrast. The image pixel value range (0-255, 8 bit) should be reasonable, with only a small protrusion at the high value range.	An **underexposed** image has low brightness (low pixel values) and low contrast (low image dynamic range), which can lead to loss of detail and compromised resolution. The pixel intensity histogram (0 - 60) spans the lower quarter of the entire image piexl range.	An **overexposed** image shows areas with high brightness, saturated pixels, and low contrast, resulting in data loss and compromised resolution. The pixel intensity histogram is cut off at the high value range (255), indicating that some pixels have saturated.
Exposure parameters directly affect image quality, and histograms are usually displayed on the microscope software for reference (as shown in the figure above). Unqualified images caused by improper parameter settings cannot be further adjusted. Retaking images may be required for downstream analysis. Note: The parameters required for tracklines and tissue differ. Refer to the imaging guide for operation.		

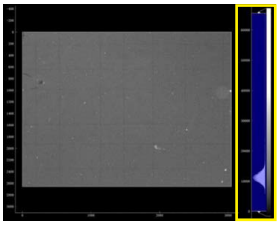
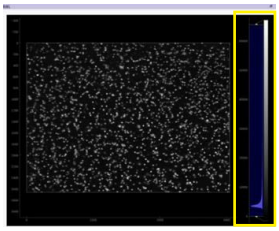
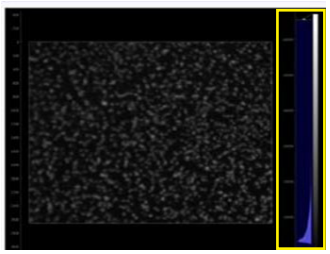
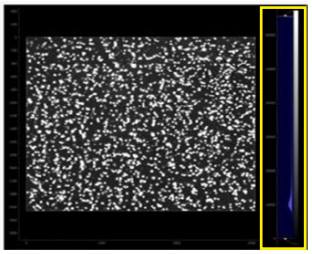
Image exposure (fluorescence)		
Qualified image	**Unqualified image**	
 Area outside the tissue  Within the tissue	 Underexposure within the tissue	 Overexposure within the tissue
A screenshot of a focus point view window selected from the background (top image) and within the tissue (bottom image); Ensure that the area outside the tissue have clear and distinct tracklines, and the stained nucleus within the tissue are clear and not exposed.	A screenshot of a focus point view window selected within the tissue showing the image and pixel statistics status when underexposed (yellow box).	A screenshot of a focus point view window selected within the tissue showing the image and pixel statistics status when overexposed (yellow box).

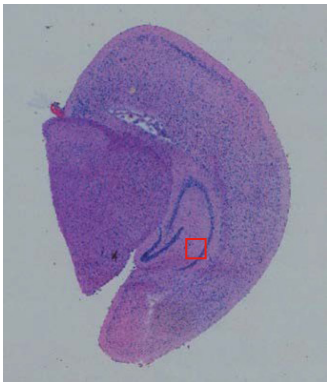
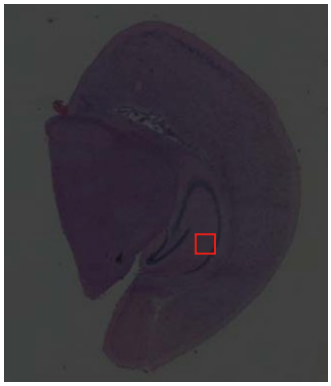
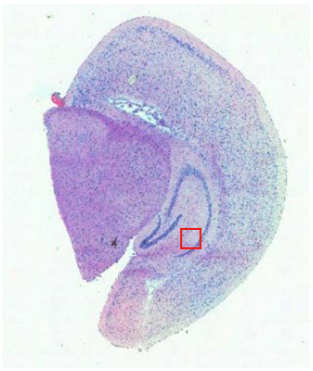
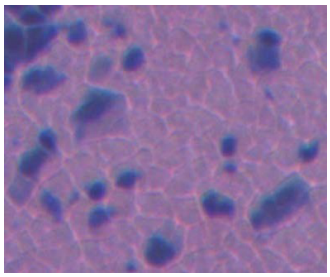
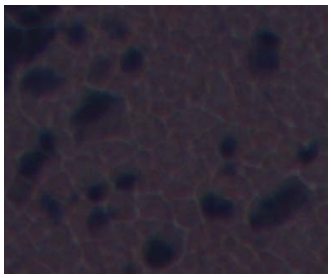
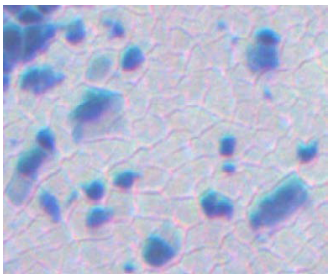
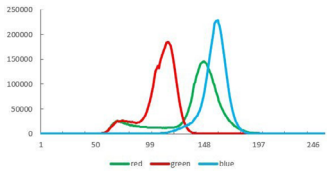
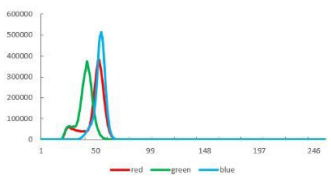
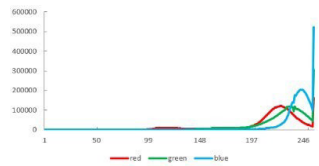
Image exposure (Brightfield)		
Qualified image	**Unqualified image**	
		
		
		
Proper exposure can provide good brightness and contrast. The image pixel value range (0-255, 8 bit) and the overall brightness value distribution should be in the middle.	An **underexposed** image has low brightness (pixel values) and low contrast (range), which can lead to inaccurate colors, loss of detail, and impaired resolution. The pixel values are distributed within the lower third of the entire pixel range (0 - 90).	**Overexposed** images have high brightness, saturated pixels (white), and low contrast, leading to inaccurate colors, loss of information, and impaired resolution. The pixel value histogram is cut off at the high value range, indicating that a large portion of the pixels have saturated and span the upper half of the pixel range (90-255).

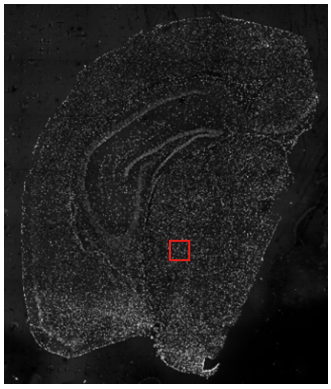
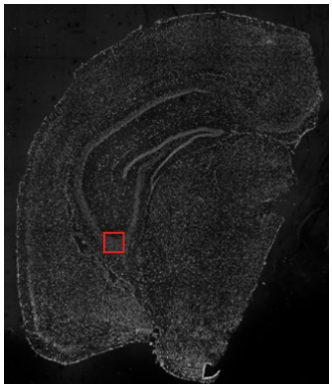
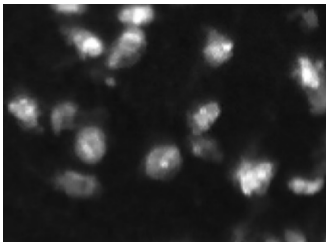
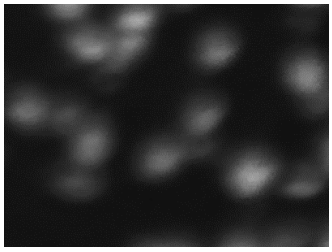
Image focus (fluorescence)	
Qualified image	**Unqualified image**
	
	
With good focus, it is possible to clearly identify the cell nuclei, as well as other morphological details and information.	Out-of-focus images will result in blurred cell nuclei and unclear morphological details.

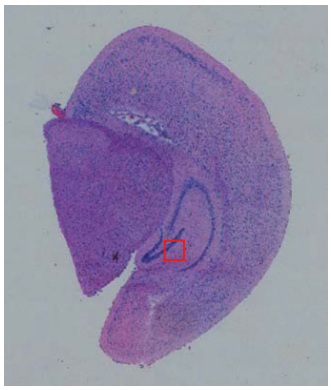
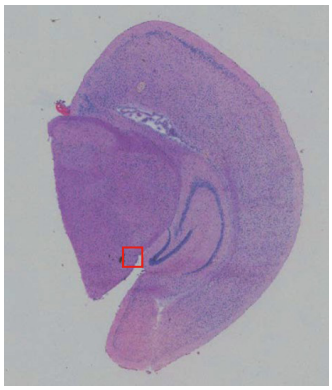
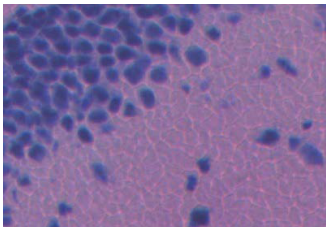
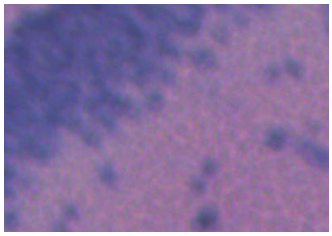
Image focus (Brightfield)	
Qualified image	**Unqualified image**
	
	
With good focus, it is possible to clearly identify the cell nuclei, as well as other morphological details and information.	Out-of-focus images will result in blurred cell nuclei and unclear morphological details.

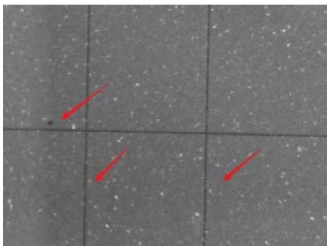
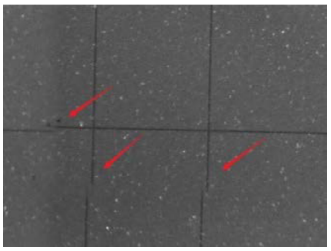
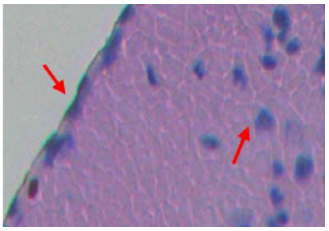
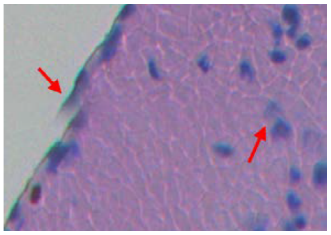
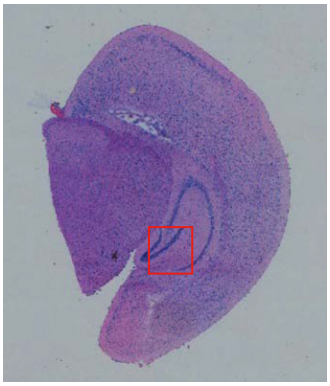
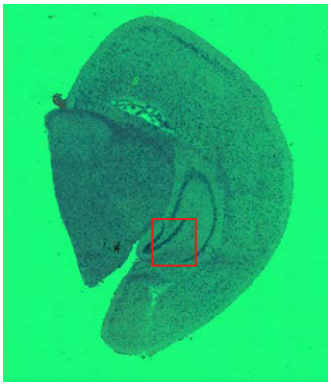
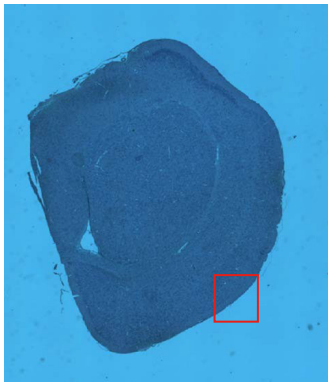
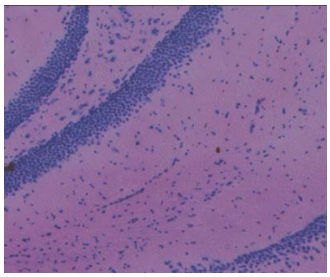
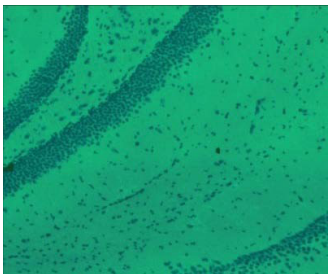
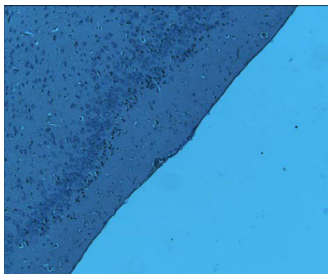
Image stitching	
Qualified image	**Unqualified image**
	
	
When stitching multiple fields of view into a large image, feature registration is necessary to correct stitching errors between adjacent fields of view caused by stage motion offsets. Above images display normal stitching with no displacement of trackline, and no displacement or double images of cells (indicated by the red arrows).	When stitching multiple fields of view into a large image solely based on stage coordinates without feature registration (or when features are not distinct), the boundaries between adjacent fields of view (red arrows) may exhibit tissue/cell displacement.

Image white balance		
Qualified image	**Unqualified image**	
		
		
In brightfield conditions, perform white balance in the blank area to obtain an image with good color tone and contrast, which can accurately reflect the staining sample conditions.	Failure to perform white balance correction produces inaccurate colors. Uncorrected color and contrast are misleading and will result in the misidentification and incorrect annotation of tissue structures.	

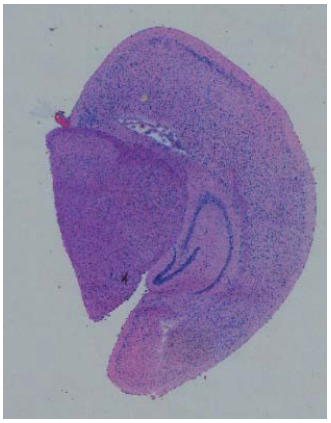
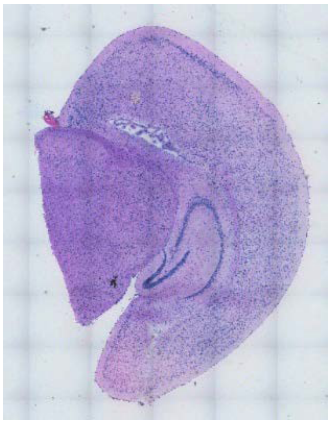
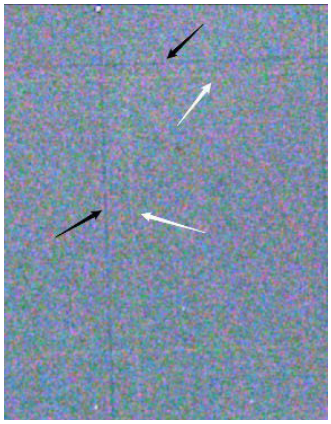
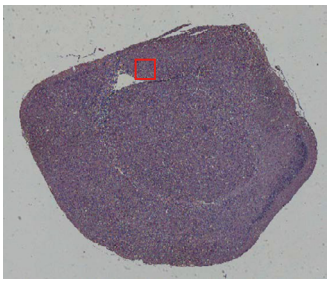
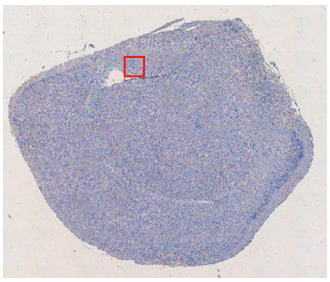
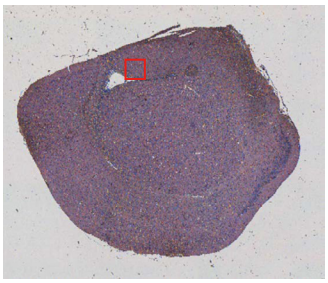
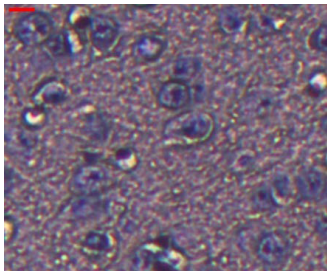
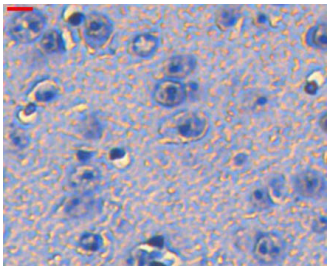
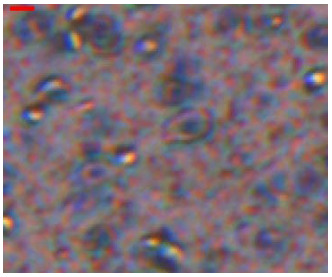
<table>
<tr><th colspan="3">Background Balance (Brightfield)</th></tr>
<tr><th>Qualified image</th><th colspan="2">Unqualified image</th></tr>
<tr><td>  </td><td>  </td><td rowspan="2">  </td></tr>
<tr><td>When stitching multiple fields of view into a large image, applying background balance can correct artifacts caused by uneven fields of view. Proper alignment and setting of the illumination path can also reduce shadow artifacts. This image has been properly balanced.</td><td>When the FOV is uneven, without correction, stitching multiple fields of view may result in shadow artifacts (stitching marks).</td></tr>
<tr><td colspan="2">These images have the same imaging field.</td><td>The background balance template should not have tracklines, and it is recommended that you use a fixed correction template. When the template has tracklines, the obtained image will show double tracklines, as shown in the above image. After adjusting the contrast of the original image, the white line is the real trackline (as indicated by the white arrow), and the black line is the false trackline (as indicated by the black arrow), the image information is abnormal, and subsequent analysis cannot be performed.</td></tr>
</table>

Image resolution (Brightfield)		
10x/0.30NA	10x/0.45NA	4x/0.13NA (Not recommended)
Objective lens resolution: 1.12 μm Image pixel size: 0.345 μm	Objective lens resolution: 0.75 μm Image pixel size: 0.345 μm	Objective lens resolution: 2.58 μm Image pixel size: 0.86 μm
		
		
The camera pixel size is 3.45 μm and the scale bar is 10 μm (red).		