STOmics

MICROSCOPE ASSESSMENT GUIDELINE FOR ImageStudio SOFTWARE

REVISION HISTORY

Manual Version:	A1	**Description**：
Kit Version:	V1.0 & V2.0	
Date:	May. 2023	· Initial release

Manual Version:	B1	**Description**：
Kit Version:	V1.0	
Date:	Oct. 2023	· 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).

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

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

NOTE: Additional operation tips and guidance.

SOLUTION: Provides a solution to the operation procedure.

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

CHAPTER 1
INTRODUCTION

STOmics Stereo-seq Solution quantifies mRNA of tissue sections followed by mapping and visualizing the transcriptomic data on an anatomical image. 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, a BGI-developed image evaluation and processing software – ImageStudio 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 "Image Quality Control" module in ImageStudio 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 4.4 and Chapter 5.3. 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

2.1. Imaging System Recommendations

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.

Index/ Parameter	Description
XY stage travel distance of microscope	At least 25*75 mm
Focusing approach	Pre-focus map and/or real-time autofocus
Brightfield light	Reflective light source (Epi-illumination)
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	High image resolution: ≥ 1800 pixels (height) and ≥2000 pixels (width)
Image bit depth	· Grayscale image: 8bit/16bit (fluorescent image) · Color image: 3*8bit/3*16bit (H&E or other brightfield image)
Exposure time	· ssDNA/DAPI: 1 milli sec - 2 sec · H&E: 0.1 milli sec - 100 milli sec
Background balance	Adjustable background balancing function
Distortion correction	Adjustable distortion correction function
Overlap ratio	Adjustable, no less than 5%
Pixel size	Maximum pixel size ≤ 5 µm/pixel
File 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

CHAPTER 3
IMAGING
GUIDELINE

3.1. Imaging System Recommendations

Table 1 shows microscope models used in developing of STOmics protocols.

Table 1 Recommended imaging systems

Manufacturer	Model/ Specification
Leica	Leica DM6-M
Zeiss	Zeiss Axio Scan Z1
	Zeiss Axio Scan 7

If none of the above microscopes is available,

a. Ensure that the microscope used fulfills hardware requirements stated in Chapter 2.
b. Perform a microscope compatibility assessment following instructions provided in Chapter 3.2.

3.2. 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 (≥ 12 bit)
· FITC filter cube (Excitation 480/30, Emission 525/50) – in microscope assessment chip and Chip T imaging
· TRITC filter cube (Excitation 545/25, Emission 605/70) – in Chip P imaging
· 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 (≥ 12 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

3.3. Input Files and Requirements for Image Registration

A tissue specimen stitched image (nucleus-stained fluorescent image) is used as an anatomical map on which the STOmics transcriptomic data are visualized. This is carried out by SAW pipelines in a step termed as `register` (image registration). Different input files are used for image registration depending on the microscope model used (Table 2).

Table 2 Input files for image registration and ImageStudio software

Microscope Model	Input file for Image Registration (nuclei-stained images only)
Zeiss Axio Scan Z1 Zeiss Axio Scan 7	Image raw file in czi
Leica DM6-M	Stitched image in TIFF
Other Microscope: · Before API development:	Stitched image in TIFF
· After API development:	All tile image in TIFF <u>or</u> Image raw file in original format

The required input files and format depend on API development method of the microscope. Please consult the corresponding STOmics FAS.

An image intended to be used for image registration must be first evaluated using Image Quality Control module within the ImageStudio software. The input image for ImageStudio software should follow the same file format indicated in Table 2. Please refer to <u>Chapter 4.3</u> and <u>5.3</u> for instructions on performing image evaluation via ImageStudio software and interpretation of the Image Quality Control evaluation results, respectively.

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
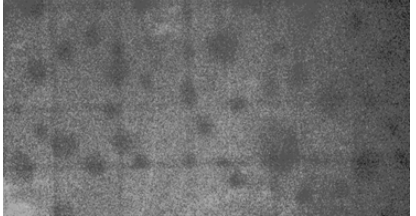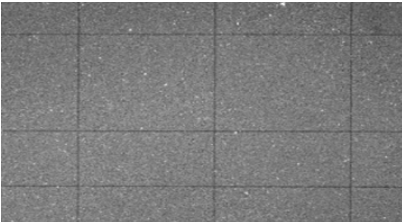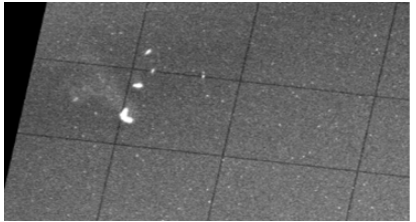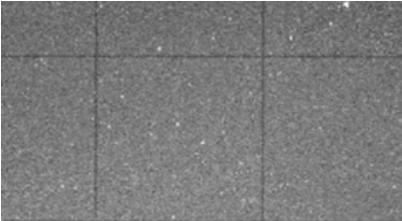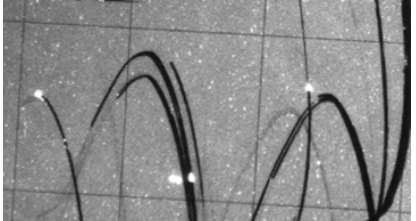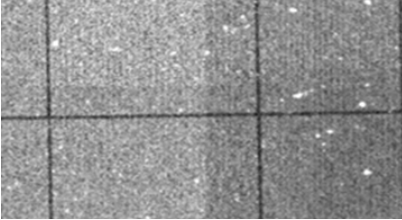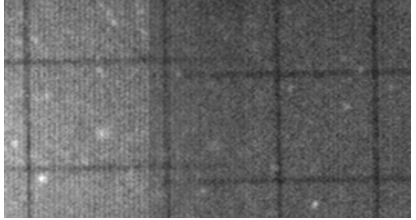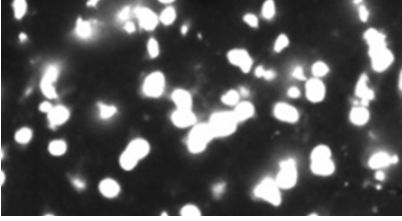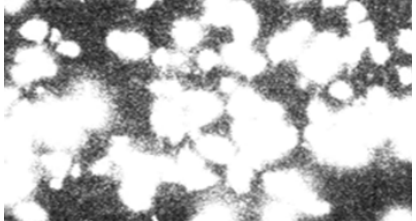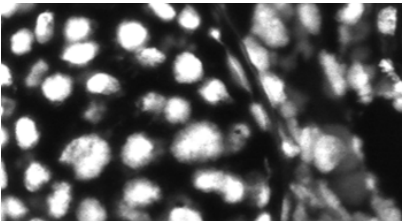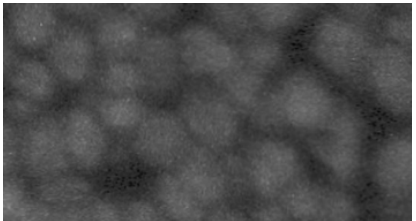
Figure shown here as an example of a high-quality mouse brain fluorescent image that has passed QC:
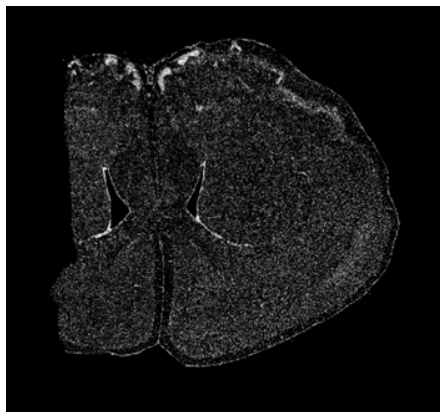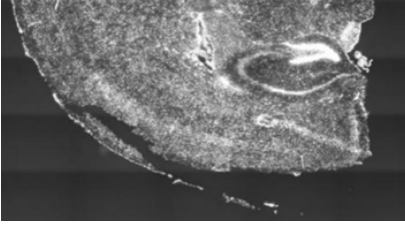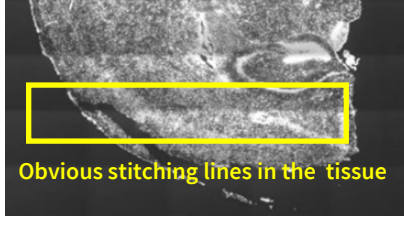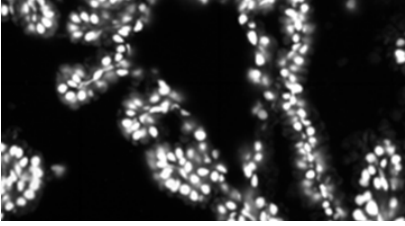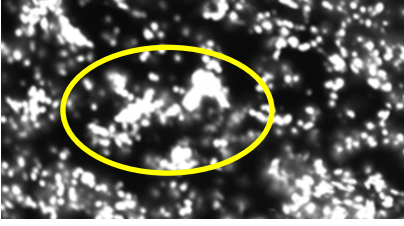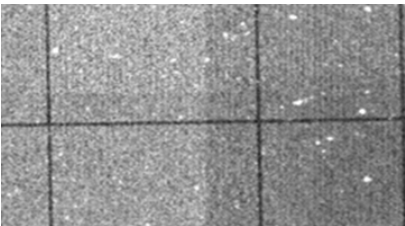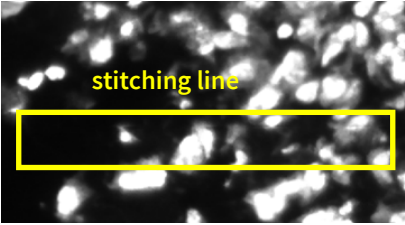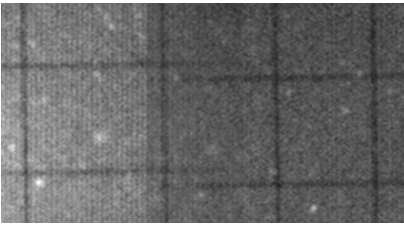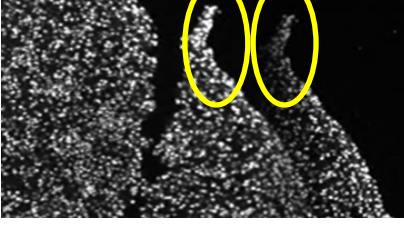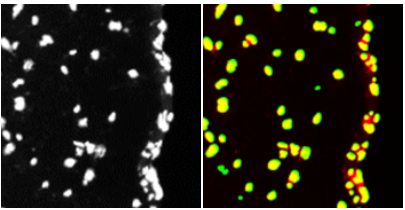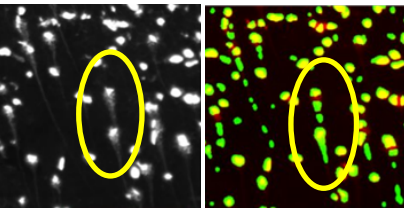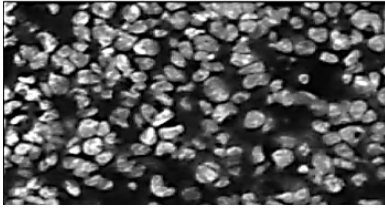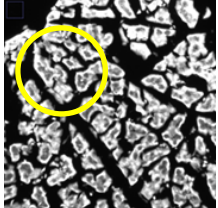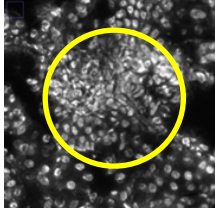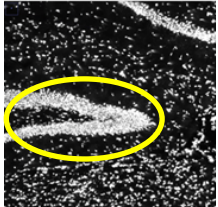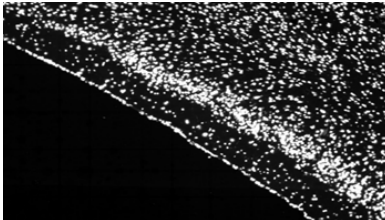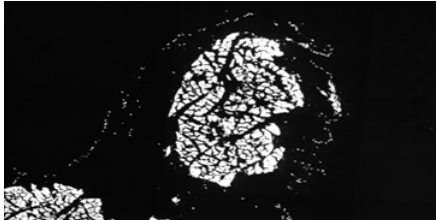
All the factors c. to f. will affect the image trackline QC score in Image Quality Control evaluation, which has a scale from 0 to 100. **A minimal score of 60 is required to pass the evaluation, the higher the score, the more accurate the image registration will be, which has a direct impact on data clustering.**

3.4. Imaging Examples

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

Issues	Correct	Incorrect
Out-of-focus tracklines		
Tracklines tilted		
Scratches		
Stitching errors (Tracklines) [1]		
Overexposure		
Out-of-focus tissue image		

Issues	Correct	Incorrect
Exposure imbalance among image tile (FOV) [2]		**Obvious stitching lines in the tissue**
Undistinguishable cell clumps[3]		
Stitching errors (Tissue image)[1]	**stitching line**	
Tailing[4]	Nucleus-stained fluorescent image (right) and transcriptomic heatmap (left)	Presence of "ghost" tails at the cells

Issues	Correct	Incorrect
Indistinct cell boundaries: joint boundaries, cell clumps and overexposure		Joint boundaries Cell clumping Overexposure of certain areas of the tissue
Clear tissue boundaries		

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.

3. The presence of cell clumps will affect the downstream single cell segmentation analysis. It usually comes with the nature of a tissue specimen and is hardly avoidable. Lowering the exposure time may help, but this could compromise the image quality of other regions of the tissue section.

4. The presence of "ghost" tails at the cells will affect the downstream cell segmentation analysis. Violent sliding of cover slip over the tissue which burst the cells, or vigorous air blowing on the tissue by a power dust remover can be the cause of this occurrence.

Imaging factors that will influence cell segmentation and cellbin results:

a. Capture images which cell boundaries can be clearly identified with naked eyes.

b. Image clarity score should be above 80.

c. Image (nuclei-stained image) MUST pass QC.

d. 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 stitching error > 5 pixel)

e. 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.

CHAPTER 4
MICROSCOPE IMAGE ASSESSMENT

The main purpose of performing a microscope image assessment using a tissue mounted Stereo-seq Microscope Assessment Chip T (STOmics, Cat. No.: 101CT111) is to assess whether the nuclei-stained image can pass the Image Quality Control evaluation before the official Stereo-seq experiment, ensuring user's microscope can produce good quality tissue-containing images (with good visibility of both trackline and tissues as well as passing the trackline QC evaluation). Good tissue-containing images of Stereo-seq Chip T (STOmics, Cat. No.: 210CT114) is important for further upstream data analysis via SAW pipelines.

4.1. Imaging System Requirements

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

a. 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.

b. Have image stitching function either via the microscope's software (automatic stitching) or STOmics image processing software, ImageStudio (manually assisted stitching, for FOV images only).

c. Have an image resolution ≥ 1800 pixels (height) and ≥ 2000 pixels (width).

d. Be able to view and save stitched images in raw file format (e.g., czi in Zeiss) and export it into TIFF.

e. 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 ImageStudio.

If none of the microscopes recommended in Table 1 (**Chapter 3.1**) is available, the prospective microscope should also have **ALL** the properties and functions listed in Table 3 or Table 4.

Table 3 Overview of fluorescent imaging system requirements

Index/ Parameter	Description
XY stage travel distance of microscope	At least 25*75mm
Focusing approach	Pre-focus map and/or real-time autofocus
Fluorescent channel	DAPI, FITC, TRITC, CY5
Objective lens	10X (NA ≥ 0.3)

Index/ Parameter	Description
Camera resolution	Camera resolution selection depends on objective lens. Please consult your microscope vendor for details
Image bit depth	Grayscale image: 8bit/16bit (fluorescent image)
Background balance	Adjustable background balancing function
Distortion correction	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
Exposure time	ssDNA/DAPI: 1 milli sec - 2 sec
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.

4.2. Pre-assessment Preparation

Materials

· Slide dryer set to 37°C or PCR thermal cycler with Stereo-seq PCR Adaptor set to 37°C
· Stereo-seq Microscope Assessment Chip T (1cm*1cm) (STOmics, Cat. No. 101CT111)
· Nuclease-free water (NF water; Ambion, Cat. No. AM9937)
· One OCT tissue block
· Staining reagent (Please select depending on your Stereo-seq application):
    · Stereo-seq Transcriptomics: Qubit® ssDNA Assay Kit (Invitrogen, Cat. No. Q10212)
    · Stereo-seq Transcriptomics mIF: DAPI Solution, 1 mg/mL (Thermo Fisher Scientific, 62248)
    · Stereo-seq Transcriptomics H&E： Hematoxylin (Sigma, Cat. No. 51275), Eosin Y, free acid （Sangon Biotech, Cat. No. A600190-0025), and Bluing Buffer, Dako (Agilent, Cat. No.CS70230-2)
· Mounting Medium:
    · Glycerol - for ssDNA and DAPI stained Stereo-seq Chip
    · H&E Mounting Medium (STOmics, Cat. No. 1000041969) - for H&E stained Stereo-seq Chip

- 5X SSC and 0.1X SSC
- 0.22 µm pore-sized filter (for filtering Hematoxylin solution)
- Power dust remover (MATIN, M-6318)
- Parafilm and dust-free paper
- 24-well plate and petri dish
- Aluminum foil or paper box

Tips and Cautions

- Avoid touching the front-side of the chip which has a shiny surface containing DNA nanoball (DNB) molecules.
- The microscope scanning area should cover the entire chip surface including the four corners.
- Contact and make an appointment with the microscope engineer of the microscope brand you are using for assistance if necessary.

4.3. Microscope Assessment Procedure

This section covers the microscope evaluation procedures for various Stereo-seq applications such as Stereo-seq Transcriptomics, Stereo-seq Transcriptomics mIF and Stereo-seq Transcriptomics H&E. Please select the appropriate evaluation procedures according to your 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 T and passing the QC, please confirm the image parameters and do not change them before the official Stereo-seq experiment.

4.3.1. Experimental Preparation

Chip Handling

a. Open the top cover of the protection box from the opening and spread it flat on the table, as shown in the picture below.
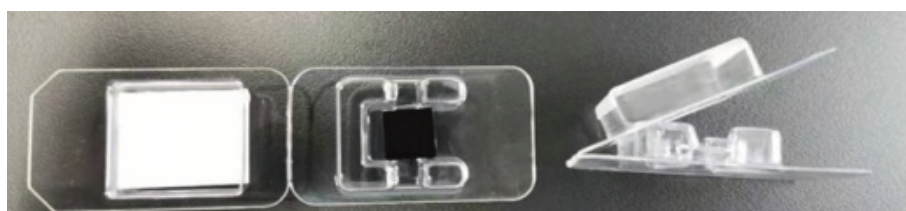
b. Insert the back end of a pair of forceps under the chip. Then, slowly rotate the forceps to give a certain force to slowly separate the chip from the double-sided adhesive then carefully detach the chip from the box without flipping the chip over.
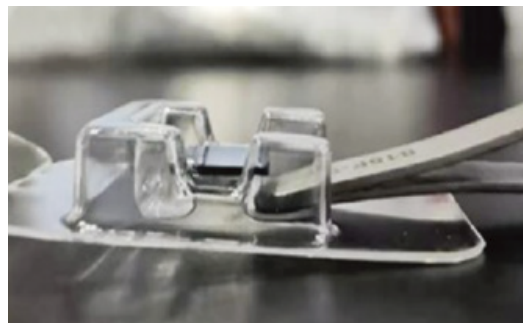
c. Take a pair of sharp end forceps and transfer the chip out of the protection box into a 24-well plate with front-side facing up.
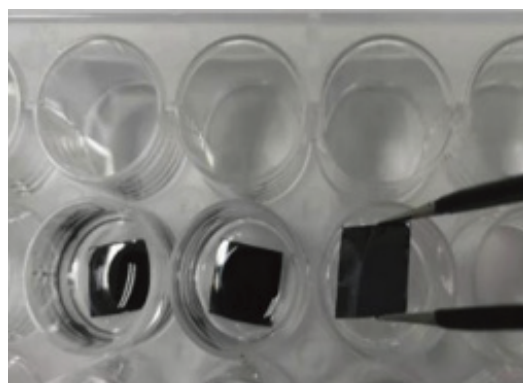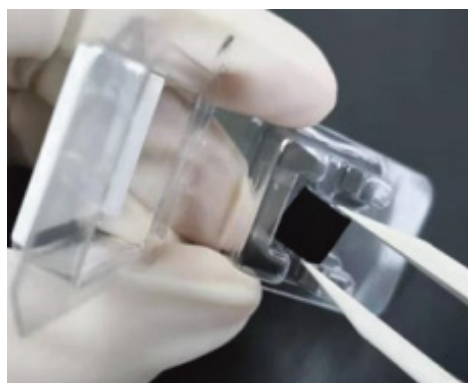
Tissue Mounted Chip Imaging Guidelines

a. Please make sure to keep the chip parallel with the edge of the stage and without tilting the chip.

b. Tissue mounting should not exceed 80% of the chip area to ensure that there is a certain amount of blank space around the tissue to reveal enough trackline areas for later image registration.

c. It is recommended to use 'manual focus' for imaging. Select the modeling points first at the four corners of the chip and then select few points within and outside of the tissue by focusing on them respectively, so as to obtain clear trackline 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.

d. Please adjust the imaging parameters to ensure that the exposure of the tissue area is well-balanced and the image, as well as the tracklines are clear. If there is any problem, please contact the microscope engineer to troubleshoot within time.

e. The imaging process should not last too long and please do not leave your chip in the same position for a long time to avoid fluorescence signal quenching.

f. Do not touch the surrounding surface during microscope scanning. Please prevent any vibration source near the microscope, such as moving and walking.

g. 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.

h. After imaging, please use ImageStudio software to QC the image immediately. If QC does not pass, please try to image again.

Cryosection Preparation:

a. Set slide dryer or PCR Thermal Cycler with PCR adaptor to 37°C in advance.

b. Set cryostat chamber temperature to -20°C and specimen disc temperature (object temperature) to -10°C~-15°C.

If the specimen disc is over-cooled, it could lead to tissue section cracking during sectioning, while sections would wrinkle when the disc temperature is too high. Optimal specimen disc temperature depends on the tissue type.

c. Place forceps, brushes, and razor blades inside the chamber for pre-cooling.

d. Take the OCT-embedded tissue sample out of the -80°C freezer to the chamber and allow it to equilibrate to the cryostat chamber temperature for 30 min.

e. Remove the sample outer covers (aluminum foil) and trim the embedded tissue block into appropriate size (sectioning area smaller than 0.9 cm x 0.9 cm).

f. By using OCT, mount the embedded tissue block onto the specimen disc/holder of the cryostat chamber.

g. Do a final trimming if necessary to ensure a good fit between the tissue section and Stereo-seq Chip T later. Now, the specimen is ready for cryosection.

Chip Preparation:

a.   Transfer a microscope assessment chip from its original packaging to a 24-well cell culture plate using a pair of forceps.

Note that every chip has a shiny surface and a matte surface, the former is the active surface of the chip and should be handled carefully. Always keep the active front-side facing upward and avoid scratches on it.

b.   Ensure the front-side of the chip is clean. If there are dust or white wavy patterns on the surface, rinse the chip with 400 µL nuclease-free water twice. After that, remove the water from the chip surface using a power dust remover and dry it on a slide dryer or Stereo-seq PCR Adaptor at 37°C for 1 minute before proceeding to the staining step below.

c.   Attach a piece of parafilm on the base of a petri dish and place the microscope assessment chip on it (front-side facing upward). This eases chip handling since it is easier to pick up a chip on a parafilm surface with forceps.
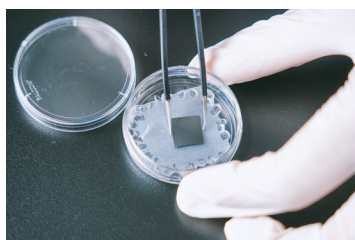
Tissue Mounting:

a.   Prepare two petri dishes and pieces of trimmed parafilm of suitable size for attaching to the petri dish bottom. Use the back-end of the forceps to gently press and fix the edges of the parafilm to the bottom of the petri dish. Place your chips on the parafilm throughout experiments to prevent chips from sliding and colliding into the wall of the petri dish.
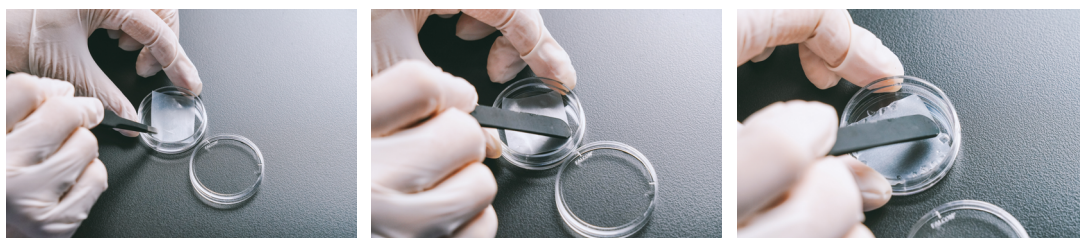
b.   Pre-cooling methanol for **5-30 min** at -20°C. Add 1 mL of methanol into one well of a 24-well plate for one Chip.

c.   Put the lid on to prevent methanol evaporation.

d.   Make sure the slide dryer or PCR Thermal Cycler with PCR adaptor has been set to 37°C.

e. Add 400 μL nuclease-free water into the well containing the chip gently along the well wall and then remove it with a pipette from the side without touching the chip surface.
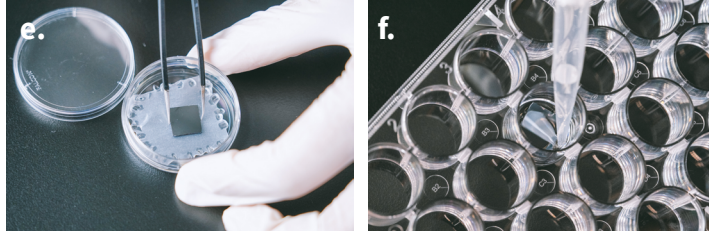
f. Remove excess water on the chip by blowing gently with a power dust remover (MATIN, M-6318) from one side of the chip at a 30~45-degree angle horizontal to the plane of the chip.
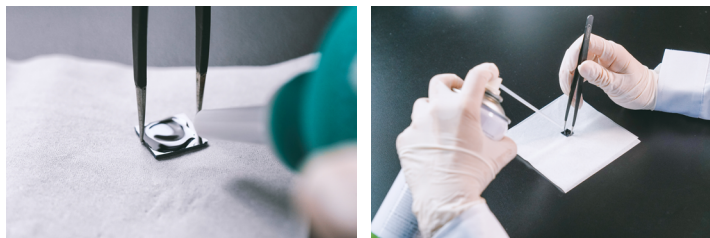
g. Dry the chip at 37°C for **1 min**. Only when the chip is completely dry and without wavy white stains, it is ready for tissue mounting.

h. Tissue mounting could be achieved via either cold method (option A) or warm method (option B).

A. Cold Method

1) Place a glass slide inside the chamber.

2) Pre-cool the chip on the glass slide inside the cryostat chamber with the front-side facing upward for **0.5~6 min**.

Prolonged cooling for over 6 min may cause mist formation on the chip surface.

3) Perform cryosection and place a tissue section on the chip carefully. Make sure the tissue section is complete and without wrinkles. Pick up the glass slide, quickly place a finger on the back of the glass slide directly under the chip for a few seconds to warm it up a little bit so that the section can adhere better to the chip.

4) Then immediately dry the chip at 37°C for **3 min** on a slide dryer.

Make sure to IMMEDIATELY dry the chip at 37°C after tissue mounting.

B. Warm Method

1) Perform cryosection, then use a soft brush to gently flatten a tissue section.

2) Hold the chip at one corner with a pair of forceps, turn it around to have its active surface facing downward, and match it onto the tissue section.

3) Lightly press the chip for a better adherence of the tissue to the chip.

4) Turn the chip again to make the tissue-mounted front-side facing upward, and immediately place it on a 37°C slide dryer for **3 min**.

Make sure to IMMEDIATELY dry the chip at 37°C after tissue mounting.

4.3.2. Stereo-seq Transcriptomics (ssDNA) Microscope Assessment

Tissue Fixation:

a.   After drying, immediately immerse the tissue-mounted chip in pre-cooled methanol prepared in section for a **30-min** fixation at -20°C. Meanwhile, make sure your microscope has been turned on, switched to FITC-mode (ssDNA) and is ready for imaging.

b.   After fixation, move the 24-well plates to a sterile fume hood.

c.   Take out the chip from the 24-well plates and absorb excess methanol on the chip from its corner and back with dust-free paper.

d.   Place the chip in the petri dish with a parafilm attached to the bottom and DO NOT close the lid.

e.   Leave the petri dish in the fume hood for **2-3 min** to let the methanol fully evaporate. While waiting, please prepare tissue fluorescent staining solution in the next section.

If the tissue turns completely white, it confirms that there is no liquid residue on the chip.

Fluorescent Staining – ssDNA:

Table 5 Stereo-seq Microscope Assessment Chip staining solution (ssDNA)

Component	Volume
5X SSC	99.5 µL
Qubit ssDNA Reagent	0.5 µL
Total	100 µL

a. Prepare a staining solution according to Table 5. Add 100 µL of tissue fluorescent staining solution onto the chip by first pipetting one droplet at each corner of the chip and then adding the rest of the staining solution to the middle to merge all the droplets. Incubate it for **5 min** at room temperature **in the dark**.

b. Gently remove the staining solution from the corner of the chip using a pipette. Wash by adding 100 µL of 0.1X SSC onto the chip surface and then remove the 0.1X SSC solution from the corner of the chip using a pipette.

c. Dry the chip further with a power dust remover by blowing gently from one side of the chip at a 30-45 degree angle horizontal to the plane of the chip.

d. Transfer the chip onto a glass slide with a pair of forceps carefully. Pipette 5 µL glycerol gently onto the center of the chip without introducing air bubbles.

e. With a pair of forceps, place one end of the coverslip onto the chip while holding the other end and then gradually lower the coverslip onto the chip. Ensure that the chip is completely covered by glycerol and the coverslip. To avoid fluorescent bleaching, IMMEDIATELY proceed to imaging.

1 µL of nuclease-free water could be added onto the glass slide first and then place the chip onto the water droplet. Water surface tension will grab onto the chip and better adhere it onto the glass slide.

Make sure the coverslip is clean without any dust or debris. Wiping with an alcohol swab or blowing with a power dust remover could be used for cleaning.

Fluorescent Staining – ssDNA:

a. Place the chip directly on the imaging stage. Mind the angle of the chip on the imaging stage, it should not be tilted.

b. Create a new folder, name it with the chip ID number and other essential information.

Only use letters, numbers, and underscores in folder naming. Special characters and spacings are not allowed.
Example: B00249A1

Imaging Guidance:

Adjust the coarse adjustment knob until the tissue appears within focus, and then use the fine adjustment knob to further fine-tune the focus until the tissue and cell contour can be clearly visualized.

Move to the tissue area first, focus on the tissue, check whether the color of the tissue area is normal. If the color appears to be off, move the focus to a blank area on the chip to adjust the white balance first.

Box selects the chip area to fully enclose the chip boundaries (should be slightly larger than the chip), scans the map under the 4X objective lens (if the microscope does not have map scanning function, skip to step 4).

After the scanning is completed, adjust the objective lens to 10X.

Further adjust the box-selected area and the edge of the chip according to the map, move to the tissue area first, then fine-tune the focus until the tissue and cell contour can be clearly visualized.

> Shift the focus to a blank area on the chip, finely adjust the focus until the tracklines on the chip can be clearly visualized, and then manually select and establish the model focal points. It is suggested to first establish 3-5 model points in the blank areas of the chip (four corners of the chip).
>
> After the establishment of the model focal points in blank areas, focus on the tissue area to establish the model focal point. It is suggested to establish 3-5 model points in different tissue areas. Once the model focal points have been established, perform a full scan using a 10X lens.

In order to ensure promising cell segmentation, make sure that both the image trackline and the tissue area are clear and the tissue area is not over-exposed.

c. Save original tile (FOV) images files and stitched images.

d. Open the ImageStudio software and the Image Quality Control functional module within the software. Upload your ssDNA-stained image and run Image QC according to the **ImageStudio User Manual** within the software.

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

4.3.3. Stereo-seq Transcriptomics mIF (DAPI) Microscope Assessment

Tissue Fixation:

a. After drying, immediately immerse the tissue-mounted chip in pre-cooled methanol prepared in section for a **30-min** fixation at -20°C. Meanwhile, make sure your microscope has been turned on, switched to DAPI-mode (DAPI) and is ready for imaging.

b. After fixation, move the 24-well plates to a sterile fume hood.

c. Take out the chip from the 24-well plates and absorb excess methanol on the chip from its corner and back with dust-free paper.

d. Place the chip in the petri dish with a parafilm attached to the bottom and DO NOT close the lid.

e. Leave the petri dish in the fume hood for **2-3 min** to let the methanol fully evaporate. While waiting, please prepare tissue fluorescent staining solution in the next section.

If the tissue turns completely white, it confirms that there is no liquid residue on the chip.

Fluorescent Staining – DAPI:

Table 6 Stereo-seq Microscope Assessment Chip staining solution (DAPI)

Component	Volume
5X SSC	98 μL
100X-diluted DAPI solution	2 μL
Total	100 μL

a.   For Stereo-seq Transcriptomics mIF application, prepare 100X-diluted DAPI solution with 5X SSC first, then prepare the staining solution according to **Table 6**. Add the rest of the staining solution to the middle to merge all the droplets. Incubate it for **2 min** at room temperature **in the dark**.

b.   Gently remove the staining solution from the corner of the chip using a pipette. Wash by adding 100 μL of 0.1X SSC onto the chip surface and then remove the 0.1X SSC solution from the corner of the chip using a pipette.

c.   Dry the chip further with a power dust remover by blowing gently from one side of the chip at a 30-45 degree angle horizontal to the plane of the chip.

d.   Transfer the chip onto a glass slide with a pair of forceps carefully. Pipette 5 μL glycerol gently onto the center of the chip without introducing air bubbles.

e.   With a pair of forceps, place one end of the coverslip onto the chip while holding the other end and then gradually lower the coverslip onto the chip. Ensure that the chip is completely covered by glycerol and the coverslip. To avoid fluorescent bleaching, IMMEDIATELY proceed to imaging.

1 μL of nuclease-free water could be added onto the glass slide first and then place the chip onto the water droplet. Water surface tension will grab onto the chip and better adhere it onto the glass slide.

Make sure the coverslip is clean without any dust or debris. Wiping with an alcohol swab or blowing with a power dust remover could be used for cleaning.

Fluorescent Staining – DAPI:

a. Place the chip directly on the imaging stage. Mind the angle of the chip on the imaging stage, it should not be tilted.

b. Create a new folder, name it with the chip ID number and other essential information.

Only use letters, numbers, and underscores in folder naming. Special characters and spacings are not allowed.

Example: B00249A1

Imaging Guidance:

Adjust the coarse adjustment knob until the tissue appears within focus, and then use the fine adjustment knob to further fine-tune the focus until the tissue and cell contour can be clearly visualized.

Move to the tissue area first, focus on the tissue, check whether the color of the tissue area is normal. If the color appears to be off, move the focus to a blank area on the chip to adjust the white balance first.

Box selects the chip area to fully enclose the chip boundaries (should be slightly larger than the chip), scans the map under the 4X objective lens (if the microscope does not have map scanning function, skip to step 4).

After the scanning is completed, adjust the objective lens to 10X.

Further adjust the box-selected area and the edge of the chip according to the map, move to the tissue area first, then fine-tune the focus until the tissue and cell contour can be clearly visualized.

Shift the focus to a blank area on the chip, finely adjust the focus until the tracklines on the chip can be clearly visualized, and then manually select and establish the model focal points. It is suggested to first establish 3-5 model points in the blank areas of the chip (four corners of the chip).

After the establishment of the model focal points in blank areas, focus on the tissue area to establish the model focal point. It is suggested to establish 3-5 model points in different tissue areas. Once the model focal points have been established, perform a full scan using a 10X lens.

In order to ensure promising cell segmentation, make sure that both the image trackline and the tissue area are clear and the tissue area is not over-exposed.

c. Save original tile (FOV) images files and stitched images.

d. Open the ImageStudio software and the Image Quality Control functional module within the software. Upload your DAPI-stained image and run Image QC according to the ImageStudio User Manual within the software.

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

4.3.3. Stereo-seq Transcriptomics H&E (Epi-illumination) Microscope Assessment

Reagent Preparation:

a.   Eosin solution: Dissolve 0.013g Eosin Y powder in 25 mL methanol and keep sealed with a parafilm until use. Prepare at least 100µL per chip.

b.   Hematoxylin solution: Prepare and filter the hematoxylin solution using a 0.22 µm pore-sized filter. Prepare at least 100µL per chip.

c.   Bluing Buffer: Prepare at least 100µL per chip.

d.   Reagent pre-cooling: Prepare a 24-well plate in advance and reserve three unused wells. Add 0.5 mL of Eosin solution into one of the wells 1 mL of Eosin solution to the other two wells. Close the lid and pre-cool the eosin solution for 5-30 min at -20°C.

Tissue Fixation & Eosin Staining (performed at –20°C)

a. After drying, immediately immerse the tissue-mounted chip in pre-cooled methanol prepared in section for a 17~27-min fixation at -20°C.

b. After fixation is done, transfer the chip to the eosin solution well and stain for 3~5 min at -20°C. The staining length can be adjusted according to the uniform coloring of the tissue and should be controlled within the range of 3~5 min.

c. Once eosin staining is done, transfer the chip back to the methanol-containing well and incubate at -20°C for another 10 min.

d. Set aside the filtered hematoxylin solution, bluing buffer, and 0.1X SSC.

e. After the second methanol fixation, transfer the chip to a new methanol-containing well and fix the tissue at -20°C for 1 min.

f. After fixation, move the 24-well plates to a sterile fume hood.

g. Place the chip in the petri dish with a parafilm attached to the bottom and DO NOT close the lid.

h. Leave the petri dish in the fume hood for 2-3 min to let the methanol fully evaporate.

If the tissue turns completely white, it confirms that there is no liquid residue on the chip.

Hematoxylin Staining and Bluing:

a. Add 100 μL of Hematoxylin Solution onto the chip by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all the droplets. Incubate at room temperature for 7 min.

b. After the incubation, slightly tilt the chip-containing petri dish while gently removing the Hematoxylin Solution from the corner of the chip using a pipette. Try to remove as much liquid as possible.

c. Add 100 μL 0.1X SSC per chip and then discard it by turning the chip sideways using a pair of forceps at an angle less than 60° while pouring out 0.1X SSC onto dust-free paper.

d. Transfer the chip back to the petri dish then repeat d. twice for a total of three washes. Try to remove as much solution as possible during the last wash by gently touching the edge of the chip with a dust-free paper to absorb the residual liquid.

e. Add 100 μL of Bluing Buffer onto the chip by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all the droplets. Incubate at room temperature for 2 min.

f. After the incubation, slightly tilt the chip-containing petri dish while gently removing the Bluing Buffer from the corner of the chip using a pipette. Try to remove as much liquid as possible.

g. Add 100 μL 0.1X SSC per chip and then discard it by turning the chip sideways using a pair of forceps at an angle less than 60° while pouring out 0.1X SSC onto dust-free paper. Try to remove as much solution as possible during the last wash by gently touching the edge of the chip with dust-free paper to absorb the residual liquid.

h. Transfer the Stereo-seq Chip Slide onto dust-free paper. Hold on to the slide with one hand and completely dry the chips further with a power dust remover in the other hand at a distance 2-3 cm away from the chip surface by blowing gently from one side of the chip at a 30-degree angle horizontal to the plane of the chip.

i. Dry the chip further with a power dust remover by blowing gently from one side of the chip at a 30-45 degree angle horizontal to the plane of the chip.

Make sure to quickly dry the chip and the surrounding surfaces completely, especially the edges of the chip. If residual liquid remains, eosin staining on the tissue might get "smudgy".

j. Transfer the chip onto a glass slide with a pair of forceps carefully. Pipette 3.5 μL H&E Mounting Medium gently onto the center of the tissue on each chip without introducing air bubbles.

k. With a pair of forceps, place one end of the coverslip onto the chip while holding the other end and then gradually lower the coverslip onto the chip. Ensure that the chip is completely covered by H&E Mounting Medium and the coverslip. To ensure good image quality, IMMEDIATELY proceed to imaging.

1 μL of nuclease-free water could be added onto the glass slide first and then place the chip onto the water droplet. Water surface tension will grab onto the chip and better adhere it onto the glass slide.
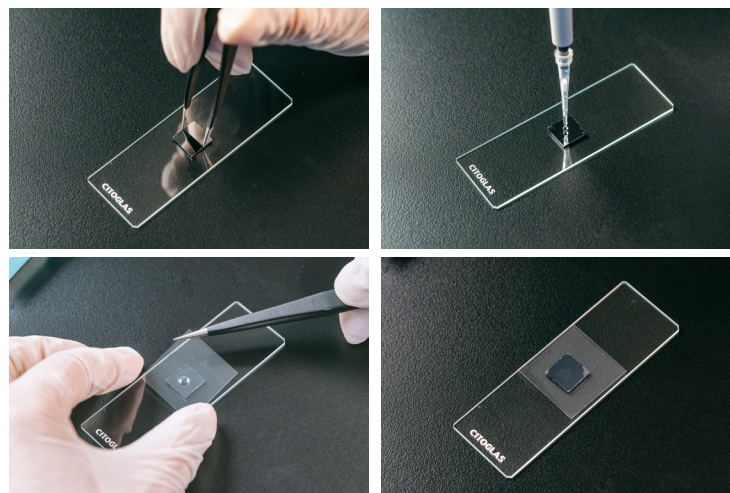
Make sure the coverslip is clean without any dust or debris. Wiping with an alcohol swab or blowing with a power dust remover could be used for cleaning.

Epi-illumination Imaging – H&E

a. Place the chip directly on the imaging stage. Mind the angle of the chip on the imaging stage, it should not be tilted.

b. Create a new folder, name it with the chip ID number and other essential information.

Only use letters, numbers, and underscores in folder naming. Special characters and spacings are not allowed.

Example: B00249A1

Imaging Guidance:

Adjust the coarse adjustment knob until the tissue appears within focus, and then use the fine adjustment knob to further fine-tune the focus until the tissue and cell contour can be clearly visualized.

Move to the tissue area first, focus on the tissue, check whether the color of the tissue area is normal. If the color appears to be off, move the focus to a blank area on the chip to adjust the white balance first.

Box selects the chip area to fully enclose the chip boundaries (should be slightly larger than the chip), scans the map under the 4X objective lens (if the microscope does not have map scanning function, skip to step 4).

After the scanning is completed, adjust the objective lens to 10X.

Further adjust the box-selected area and the edge of the chip according to the map, move to the tissue area first, then fine-tune the focus until the tissue and cell contour can be clearly visualized.

Shift the focus to a blank area on the chip, finely adjust the focus until the tracklines on the chip can be clearly visualized, and then manually select and establish the model focal points. It is suggested to first establish 3-5 model points in the blank areas of the chip (four corners of the chip).

After the establishment of the model focal points in blank areas, focus on the tissue area to establish the model focal point. It is suggested to establish 3-5 model points in different tissue areas. Once the model focal points have been established, perform a full scan using a 10X lens.

In order to ensure promising cell segmentation, make sure that both the image trackline and the tissue area are clear and the tissue area is not over-exposed.

c. Save original tile (FOV) images files and stitched images.

d. Open the ImageStudio software and the Image Quality Control functional module within the software. Upload your H&E-stained image and run Image QC according to the ImageStudio User Manual within the software.

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

## 4.1. Interpretation of Image QC Evaluation Results

Please download and install the latest version of ImageStudio software. Please see **Chapter 5.1** for software download link; **Chapter 5.2** for installation guidelines.

Perform image QC via the "Image Quality Control" module as described in **Chapter 5.3**. Depending on the type of microscope used, different image files and formats need to be entered into the software, as shown in **Table 2** of **Chapter 3.3**.

4.4.1 Image trackline QC score

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
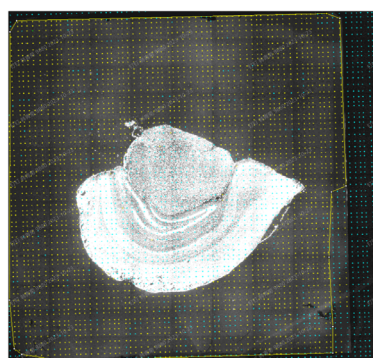
Demonstration of Image trackline QC calculation process

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

4.4.2   Image clarity score (applicable for ssDNA & DAPI)

Image clarity score is calculated using a trained deep learning model which slices the input image, 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.

CHAPTER 5
INTRODUCTION TO IMAGESTUDIO

5.1. General Information

STOmics ImageStudio is an offline image processing software that is specifically designed to provide multiple functionalities for Stereo-seq Chip images. It includes easy-to-use functional modules for microscope image quality evaluation and manual adjustment (image stitching, tissue segmentation and cell segmentation). Image quality evaluation mainly evaluates the clarity and stitching quality of an image by referring to the background tracklines on the Stereo-seq Chip in order to meet requirements for further downstream bioinformatics analysis. When automatic stitching or segmentation algorithms can not satisfy the needs for further downstream analysis, manual adjustment modules can provide manual tools for image processing.

Software logo:

Computer system requirements:

· Hardware requirement: memory of at least 16G.
· Operating system: Windows 10 - 64 bit
· Connection requirement: Not necessary unless image uploading to a computer cluster or cloud is required.

Software download or update links:
https://en.stomics.tech/products/stomics-software/stomics-offline-software

5.2. Installation Instructions

You can install different functional modules based on your requirements. The installation instructions are as follows:

5.2.1. First Installation

⚠️   ⚠️   **The installation instructions and screenshots of this manual use V1.0.0 (beta) as an example. Other versions are the same as V1.0.0 (beta).**

a.   Download **'ImageStudio_1.0.0_beta.exe'** installation package from links given in section 1.3. The software installation might get blocked, simply click on the '…' button and select 'Keep' to recognize the software installation package as a trusted file.
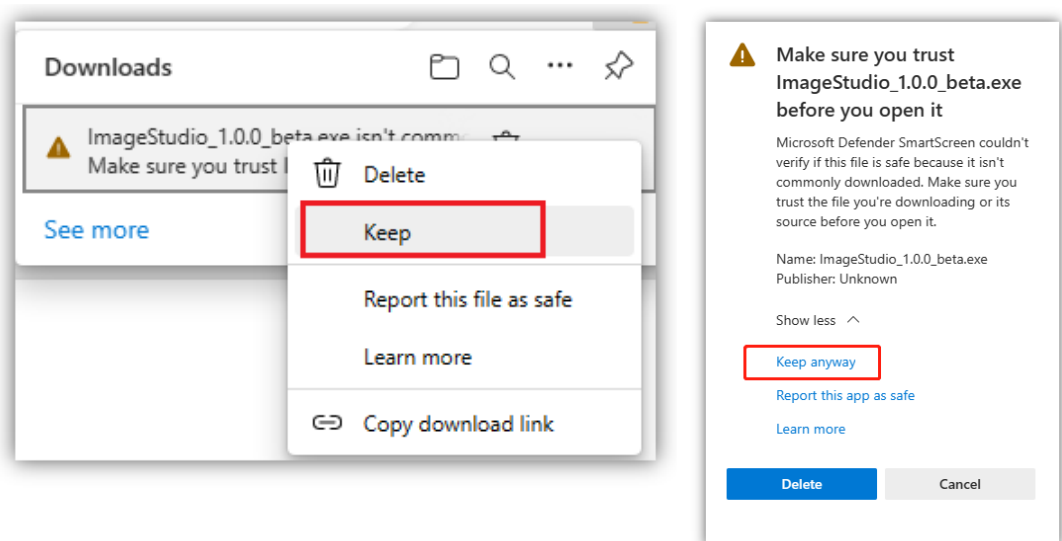
b.   Double click the downloaded file **'ImageStudio_1.0.0_beta.exe'**
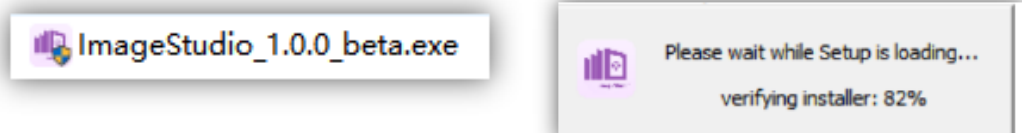
c.   Select a destination location. (The system creates the ImageStudio folder on the D: Drive.)

⚠️
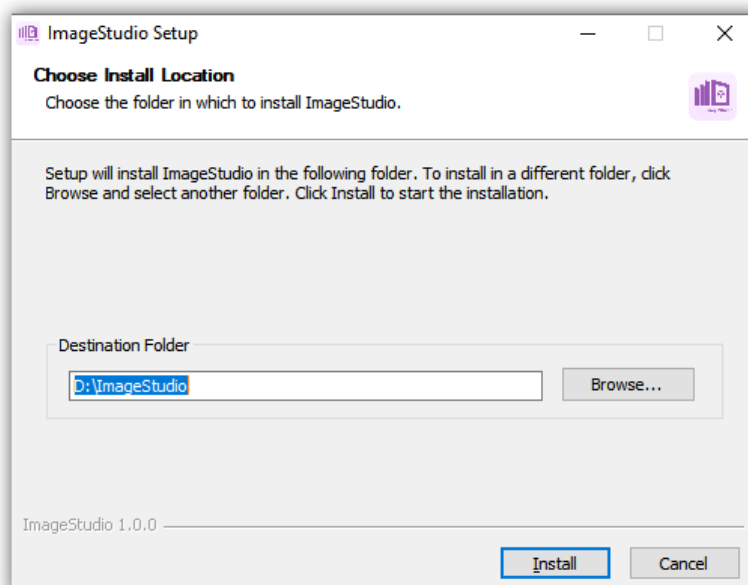
⚠️ **Changing the default installation directory is not recommended, If necessary, please do not install into the C: Drive as errors might occur due to lack of administrative permission.**

⚠️ **Please ensure there is enough space on D: Drive. It is recommended to have at least 20G storage.**

d. Click **"Install"**
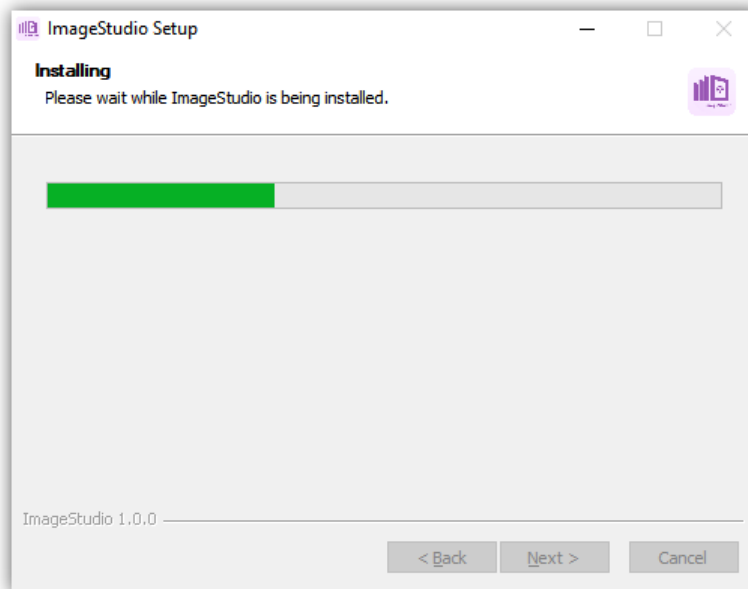
e. Once the installation is completed, select the option **'Run ImageStudio'** and click **'Finish'** to launch the program.
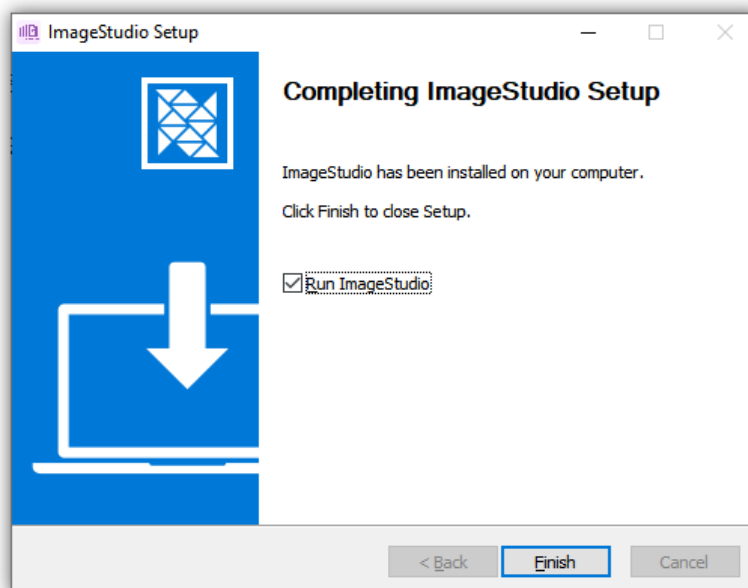
f. After the installation, double-click the shortcut icon on the desktop to launch.
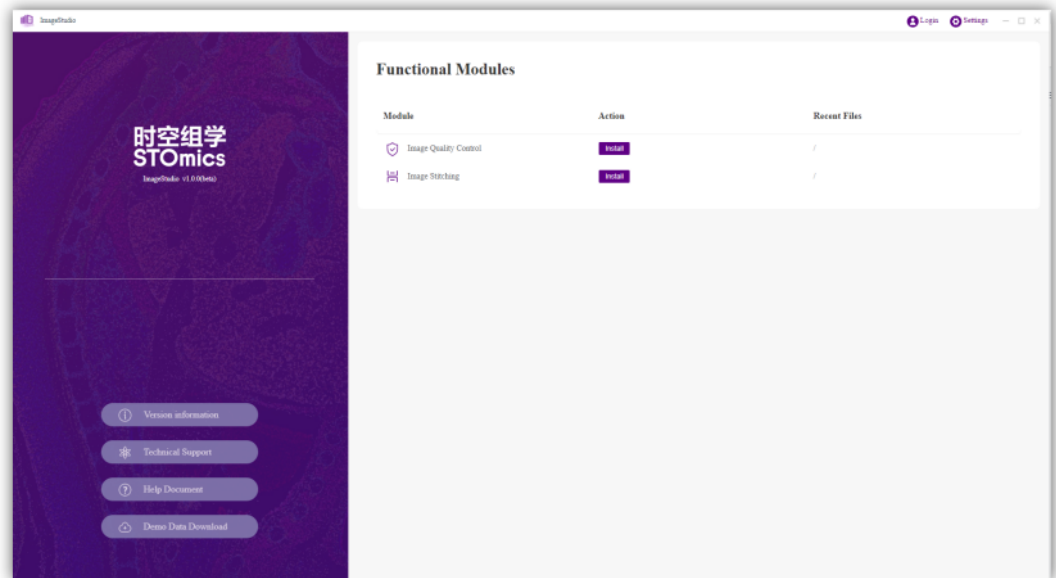
g. Click to install the corresponding functional module. Here, we use **'Image Quality Control'** as an example:
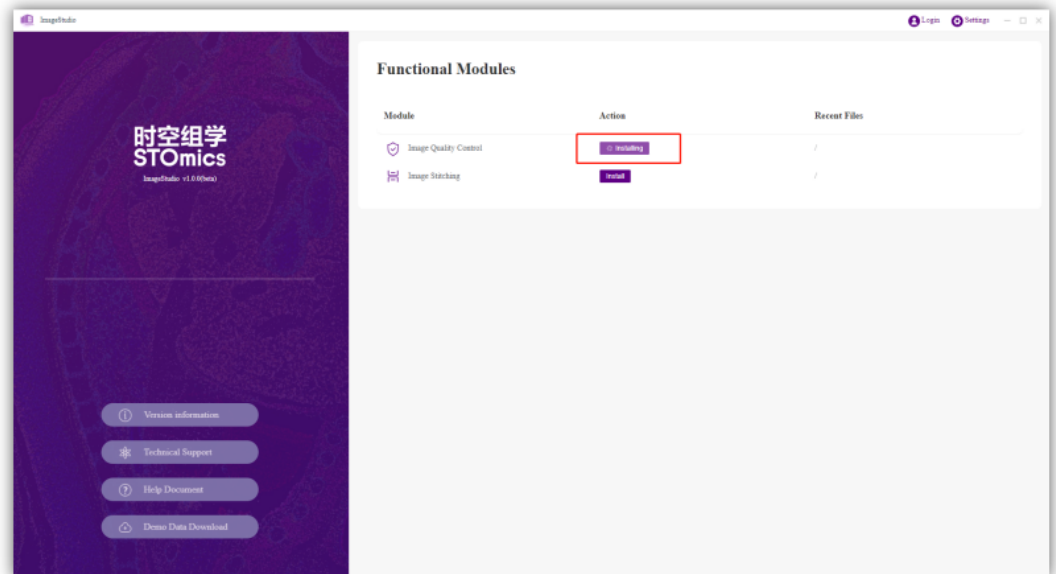
⚠️ **Do not install all modules at the same time. Install the next functional module once the previous one has been finished.**

h. After the functional module has been installed, click **'Start'** to access the module's interface.
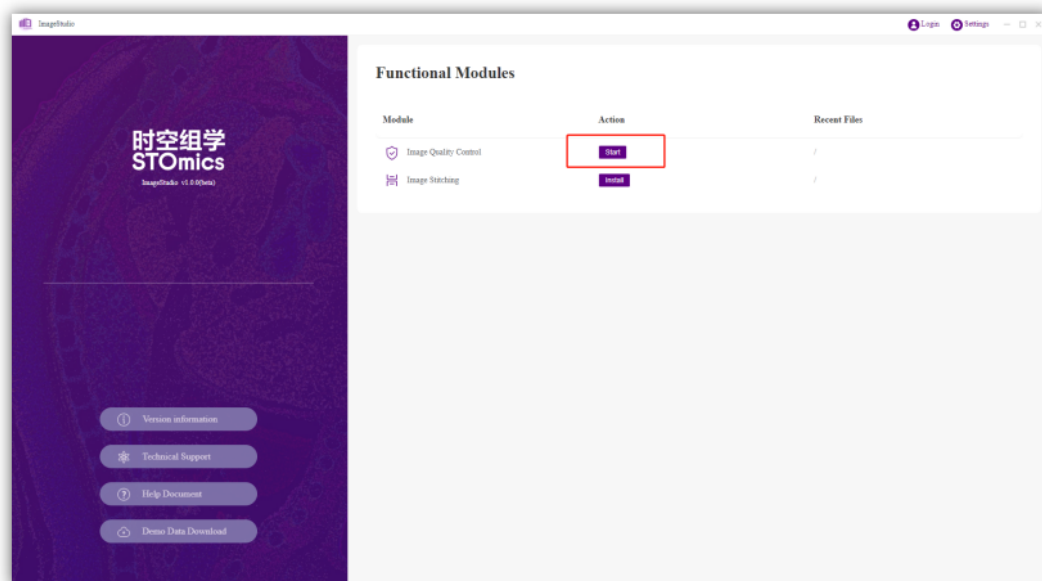
i. Repeat step g. to install other functional modules.

5.2.2. Software Update

a. Once the installation is completed, open **'Settings'**.
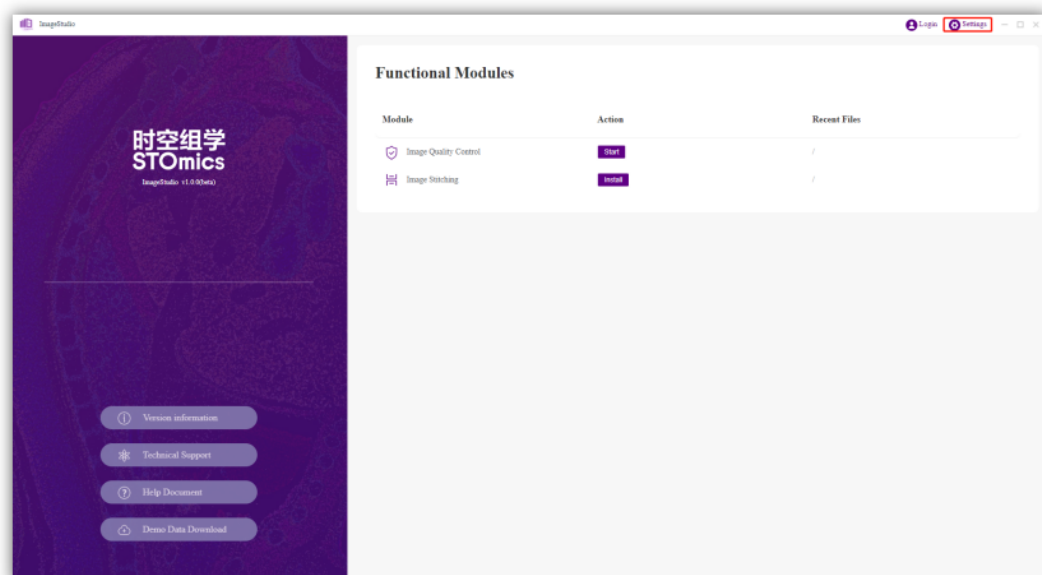
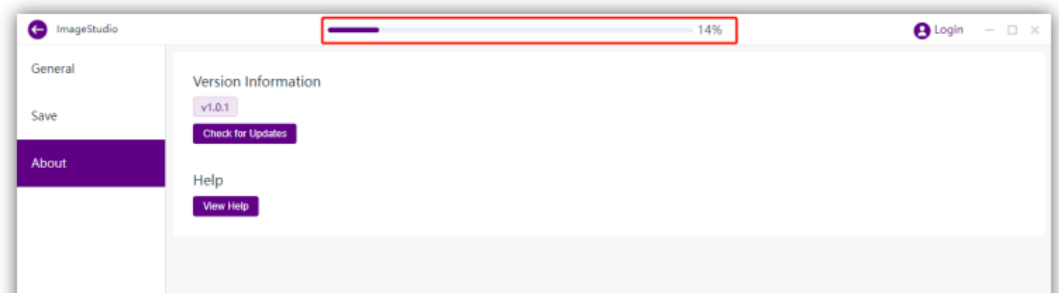b. Click About and check for updates. The system can automatically check for updates with stable internet connection.
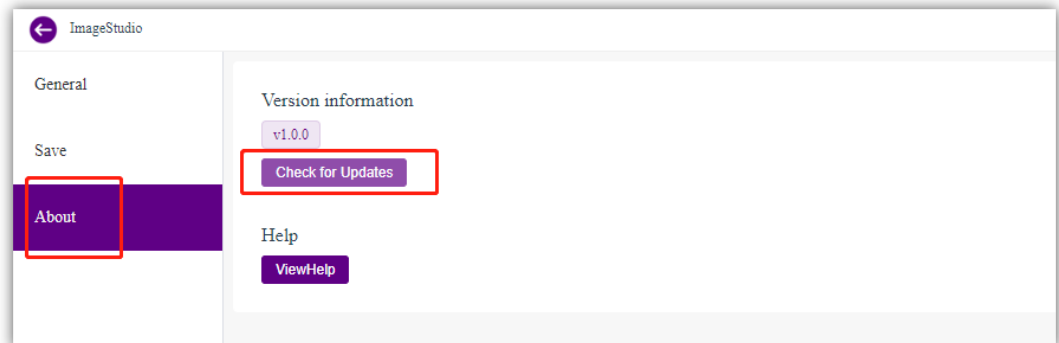
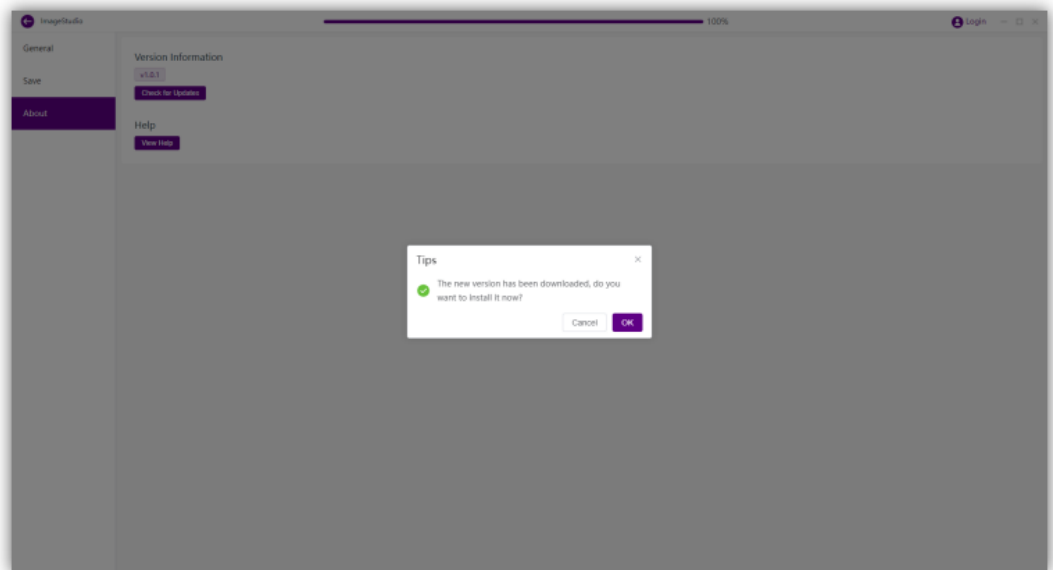c. If there is no version to be updated, the following information will display.
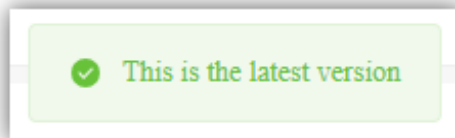
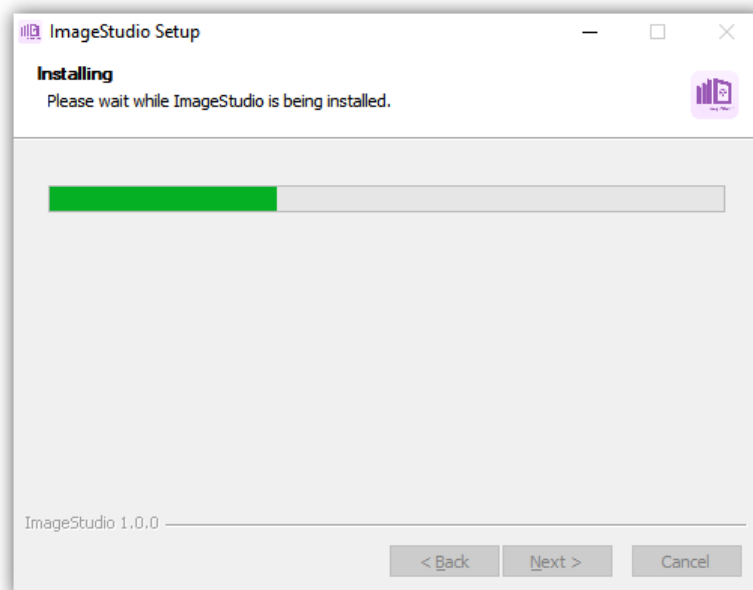d. If new version is available for update, a pop-up message will display.
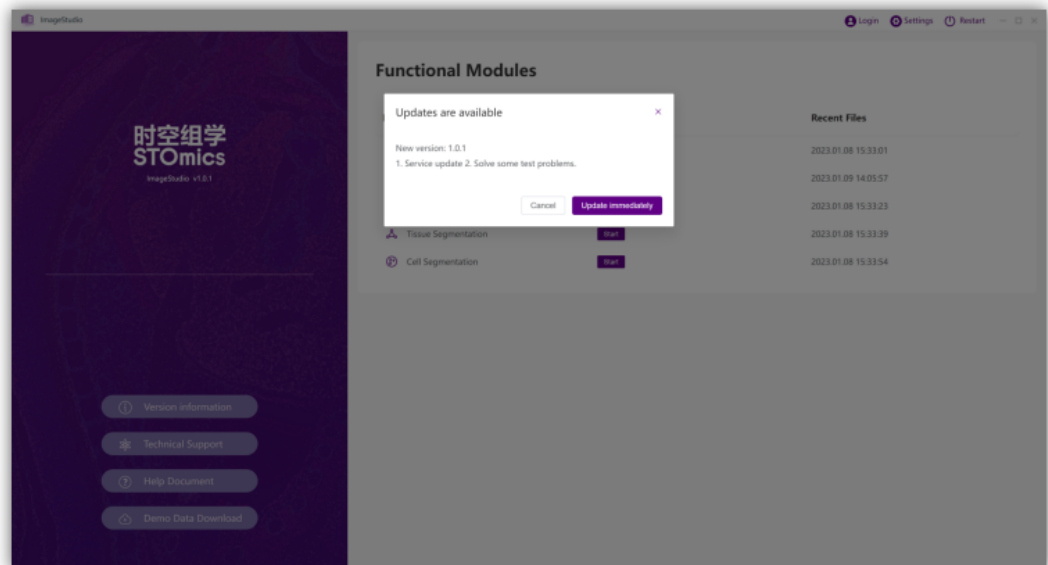
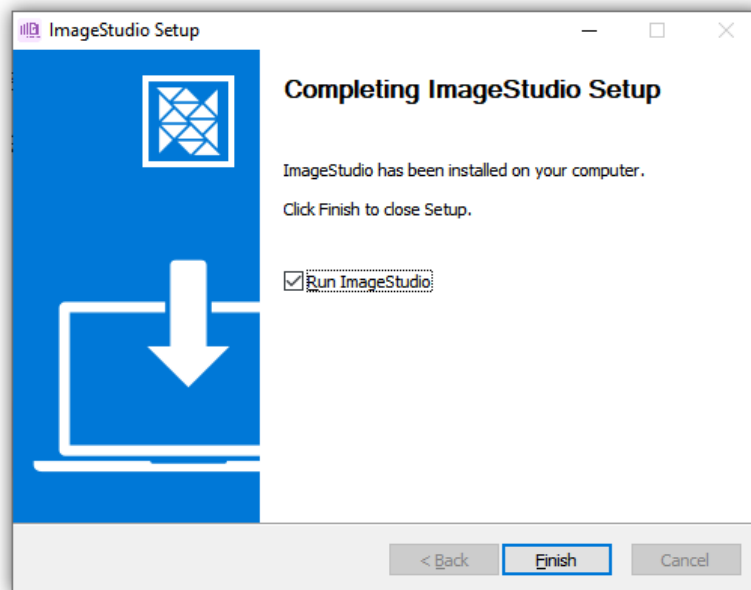e. Click **'Update Immediately'** to download the installation package.

f.   Once complete, click **'OK'**.


g.   Enter the Installation interface.

h. Now the update has been completed.

Make sure to update the software under internet connection. If internet connection is unavailable, contact your local FAS for assistance.

5.2.3. Software Uninstallation

a. Open the system's control panel or find **'Uninstall ImageStudio.exe'** under the program installation directory (Default: drive D:/ImageStudio). (Users can save the installation package of the latest version as a backup.)
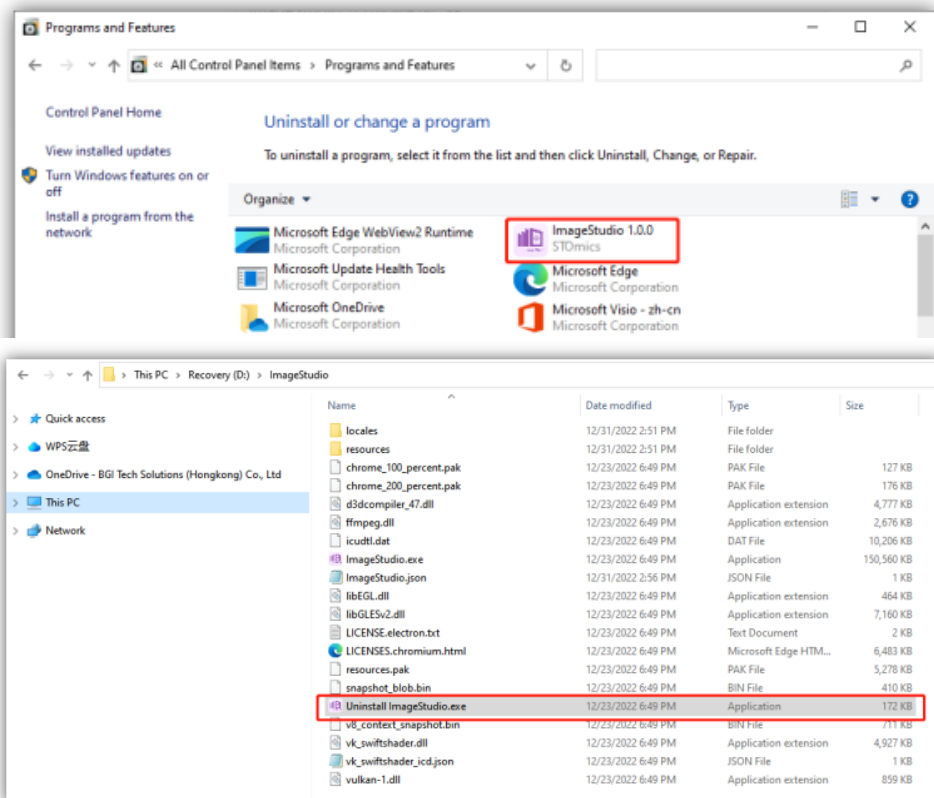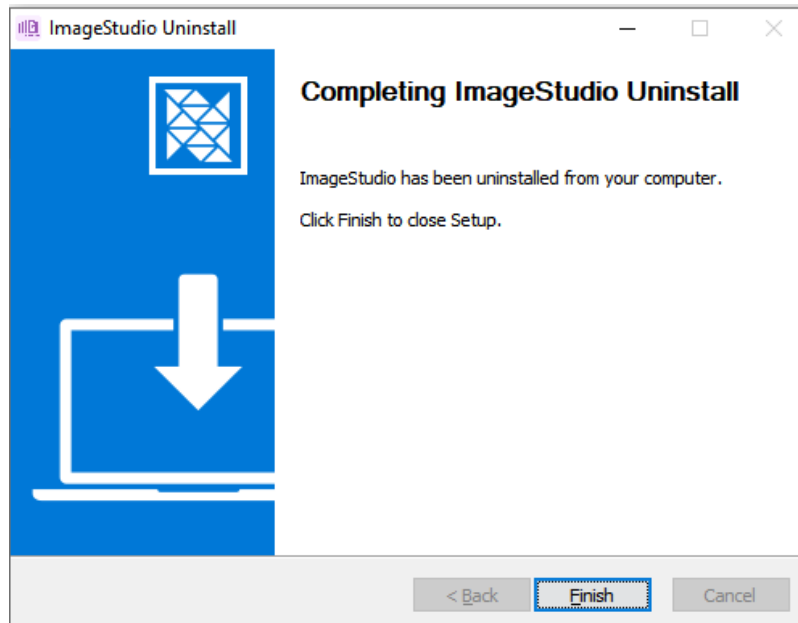

b. If a pop-up prompts that the program is occupied, click **"OK"** to close the ImageStudio program window.
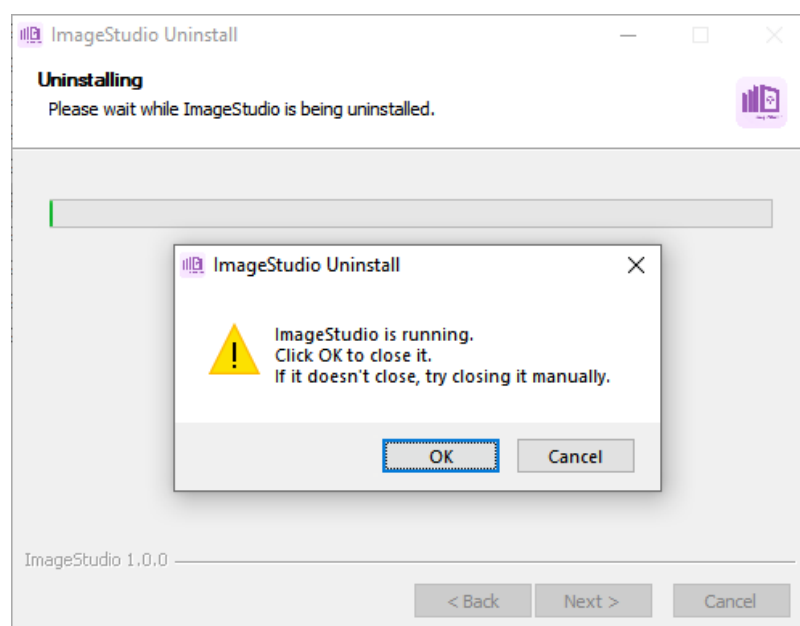
c. Click **'Next'** to uninstall.
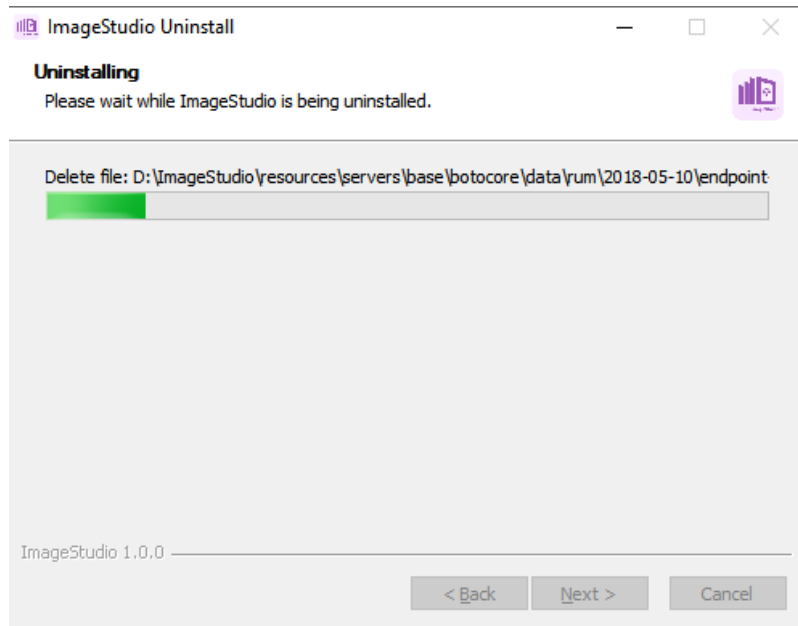
d. Click **'Next'** to complete the uninstallation process.

5.3. Additional Interpretation of Image Quality Evaluation Results

5.3.1. 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".
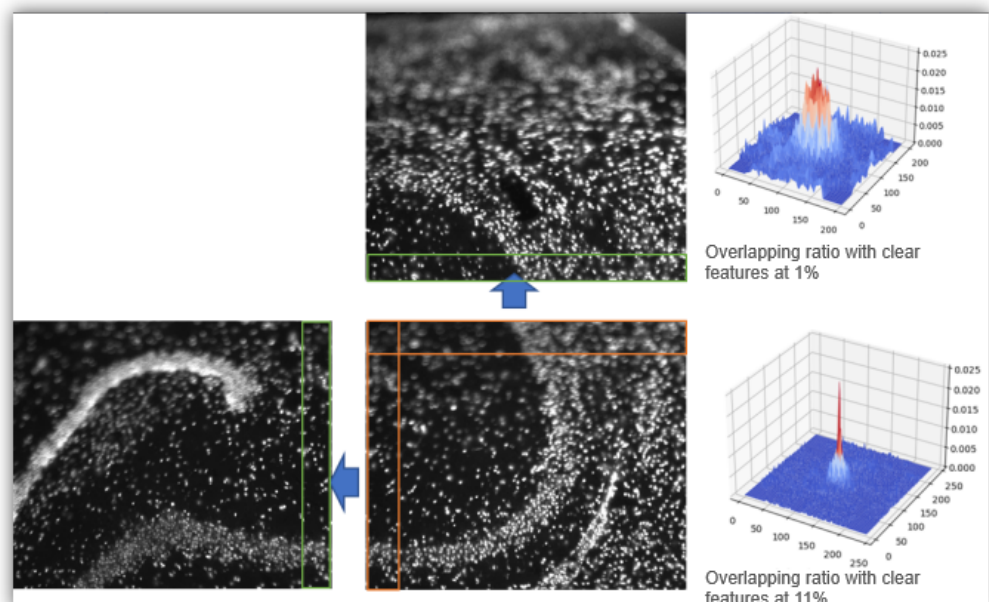
Example of overlapping area with clear features. The features of the overlapping area are clear when the ratio is greater than 5%.

5.3.2. Image Calibration Assessment Scoring Instructions

Image calibration assessment: It utilized similarity calibration algorithm to evaluate the offset between DAPI and it's corresponding IF images. The passing criteria is 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.

5.4. ImageStudio Software

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