

**GUIDEBOOK for
ImageQC SOFTWARE,
MICROSCOPE ASSESSMENT AND IMAGING**

Manual Version: A5



Revision History

Manual Version: A0

Software Version: V1.0.1

Date: Aug. 2021

Description: Initial release

Manual Version: A1

Software Version: V1.0.6

Date: Nov. 2021

Description:

- Stitched large image instruction
 - Remove instruction (ImageQC is not responding)
 - Modify chip number and experimenter instructions
 - Added the exception handling instruction for long QC time
 - Added installation directory suggestion
-

Manual Version: A2

Software Version: V1.0.7

Date: Dec. 2021

Description:

- Updated the software screenshots up to date
 - Added install mode suggestion
 - Deleted the exception handling instruction for long QC time
 - Added the handling instruction for unexpected error
 - Update instructions for chip staining, fluorescence imaging, and ImageQC in the 2.5 Imaging Test Procedure
-

- Added chip scratch and excessive inclination of trackline picture examples
-

Manual Version: A3

Software Version: V1.0.8

Date: Apr. 2022

Description:

- Combine microscope assessment instructions, imaging guidelines, ImageQC software and API development in one guidebook
 - Added more details about input files used for ImageQC and image registration in SAW
 - Added more image examples and advice to avoid poor quality images
 - Added introduction of ImageQC software
 - Added requirements for microscope Application Programming Interface (API) development
-

Manual Version: A4

Software Version: V1.0.8

Date: July. 2022

Description:

- Added pipelines tailoring ImageQC output files of a stitched image to SAW.
- Update illustration diagrams of the ImageQC software interface.
- Added a new evaluation score – QC blur score to evaluate the cell boundary (not determining the evaluation final pass/fail result)

- Compatible with the short code version of Stereo-seq chip numbers (SN number).
- Compatible with mouse brain and macaque tissue czi data; cancelled reading via a second channel.
- The local tar.gz and json files will not be deleted or moved after successful uploading via rsync, in this version.
- New ImageQC scoring criteria description for the assessment process.

Manual Version: A5

Kit Version: V1.1.3

Date: Sep. 2022

Description:

- Added image stitching evaluation function.
- Added explanations of imaging factors that will affect further cell segmentation and cellbin.
- Described criteria for cell segmentation
- Updated microscope evaluation process.
- Updated chip name to Chip T (chip size * chip size).
- Removed restrictions on ssDNA staining image rotational angle.
- Adapted to various channels of czi format.
- Changed blur score and trackline score display to percentage system.
- Support saving information in QC output excel, txt, json files to ipr files, and uploading only ipr files and raw data.

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

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NOTE: Additional operation tips and guidance.



SOLUTION: Provides a solution to the operation procedure.



CAUTION: Proceed with extra care; improper handling may cause failure or biased results.

CHAPTER 1

INTRODUCTION

Introduction

The STOmics SpatioTemporal Gene Expression Analysis 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 procedure. Additionally, a BGI-developed image evaluation software – **ImageQC** is also introduced in this guidebook.

CHAPTER 2

IMAGING GUIDELINES

2.1. Imaging System Recommendations

Table 1 shows microscope models used by BGI Research in developing 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,

- Ensure that the microscope used fulfill requirements stated in section 3.1.
- Perform a microscope compatibility assessment following instructions provided in section 3.2.
- After performing the microscope compatibility assessment, we strongly recommend the user to send the stitched image (TIFF/ PNG) and ImageQC output files (json and tar.gz) to our field application scientist (FAS). A further evaluation will be done to determine if an application programming interface (API) development is required to tailor the microscope's software to the BGI-developed data analysis pipelines, namely Stereo-seq Analysis Workflow (SAW). This will greatly improve the accuracy in anatomical/ spatial mapping of gene expression data later. However, we will need the user to connect our FAS to the manufacturer's microscope engineer to obtain some information and stitching parameters of the microscope for our R&D team to develop an API.



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

2.2. Imaging Configurations

Recommended Imaging Configuration:

Recommended Fluorescence Configuration:

- Light source with a wavelength range of 380 - 680 nm
- Monochrome camera (\geq 12 bit)
- FITC filter cube (Excitation 470/40, 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 μ m
- Exposure time 1 milli sec - 2 sec

2.3. 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 ImageQC software

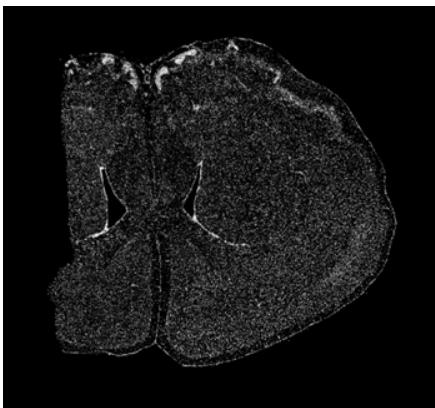
Microscope Model	Input file for Image Registration
Zeiss Axio Scan Z1	Image raw file in czi
Zeiss Axio Scan 7	
Leica DM6-M	Stitched image in TIFF/ PNG
Other Microscope:	
• Before API development:	Stitched image in TIFF / PNG
• After API development:	All tile image in TIFF / PNG or Image raw file in original format

An image intended to be used for image registration must be first evaluated using ImageQC software. The input image for the ImageQC software should follow the same file format indicated in Table 2. Refer to section 4.3 and 4.4 for instructions to perform image evaluation via ImageQC software and interpretation of the ImageQC evaluation results, respectively.

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

- a. High image resolution: ≥ 1800 pixels (height) and ≥ 2000 pixels (width) 
- b. The imaged tissue nuclei should be clear and within focus
- c. Tracklines on the chip should also be clear and within focus 
- d. No stitching errors
- e. Avoid photobleaching
- f. Tissue area should not exceed 80% of the chip area

All the factors c. to f. will affect the image trackline QC score in ImageQC 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 which has a direct impact on data clustering. Further, the trackline QC score of a microscope assessment chip image is usually higher than that of a Chip T (1cm*1cm) image because most of the tracklines on the Chip T (1cm*1cm) are covered by the tissue.**



Nucleus-stained fluorescent image of a mouse brain coronal section which passed the ImageQC

2.4. Tissue Mounted Chip Imaging Guidelines

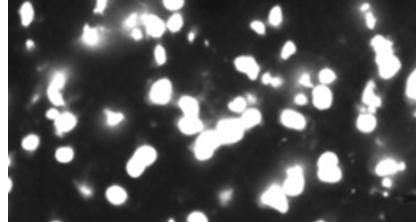
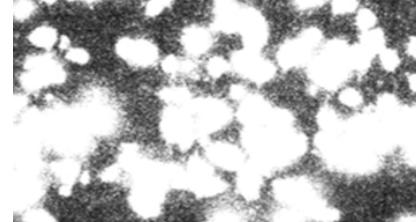
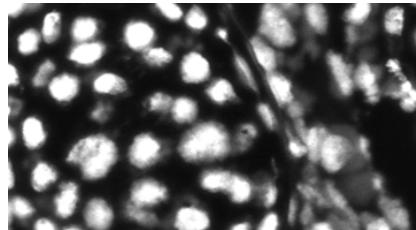
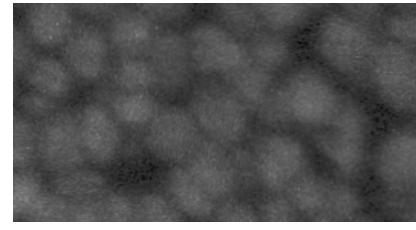
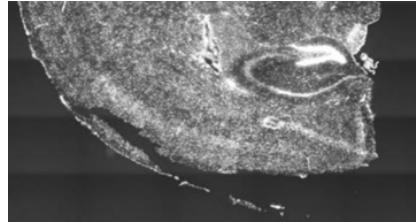
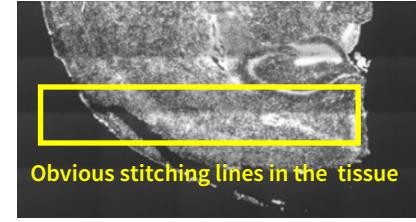
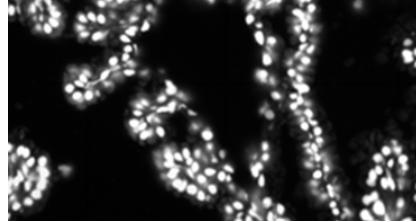
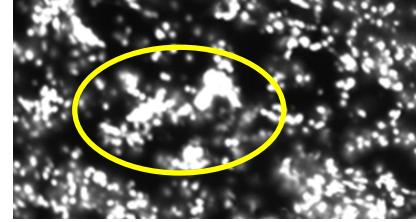
- a. Please try to ensure that the tissue size is within 0.9cm x 0.9cm x 2cm. Tissue mounting should not exceed 80% of the chip area to ensure that there is a certain amount of blank space around the tissue to reveal enough trackline areas for later image registration.
- b. Please make sure to remove as much staining solution as possible and clean the chip with 0.1x SSC to prevent excessive salt stains on the chip surface affecting the imaging quality. Make sure to completely dry the 0.1x SSC solution as well using a dust-free paper to first absorb the solution from the side of the chip, and then gently blow dry the residual liquid with a power dust remover.
- c. Before glycerol mounting, please check whether the surfaces of the cover-slide and coverslip are clean, if there are impurities, clean them with 75% ethanol or blow with a power dust remover. In addition, please cover the slide slowly from one side of the chip to the opposite side to avoid air bubble formation during glycerol mounting.
- d. The imaging process should not last too long and please do not leave your chip at the same position for a long time to avoid fluorescence signal quenching.
- e. 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.
- f. After imaging, please use ImageQC software to QC the image immediately. If QC does not pass, please try to image again.

2.5. Imaging Examples

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

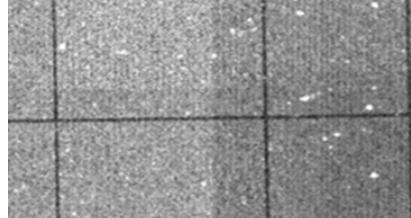
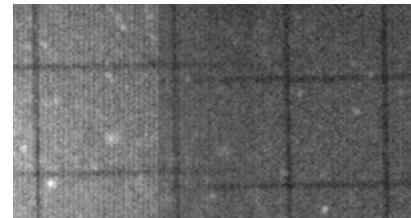
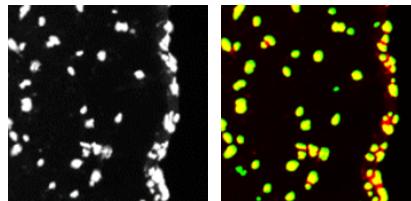
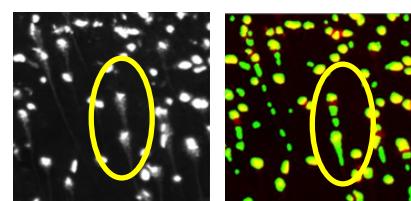
Issues	Correct	Incorrect
Out-of-focus tracklines		
Scratches		
Tracklines tilted		
Stitching errors (Tracklines) ¹		

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

Issues	Correct	Incorrect
Overexposure		
Out-of-focus tissue image		
Exposure imbalance among image tile (FOV) ²		 Obvious stitching lines in the tissue
Undistinguishable cell clumps ³		

²The 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.

³The 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.

Issues	Correct	Incorrect
Stitching errors (Tissue image) ¹	 <p>Adjacent FOVs are precisely stitched</p>	 <p>Repetition due to stitching errors</p>
Tailing ⁴	 <p>Nucleus-stained fluorescent image (right) and transcriptomic heatmap (left)</p>	 <p>Presence of “ghost” tails at the cells</p>

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

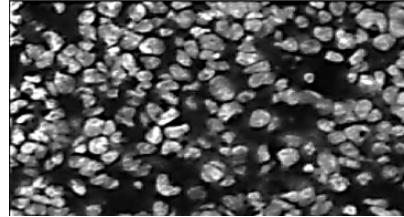
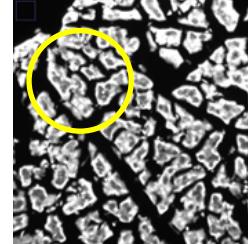
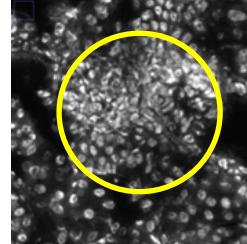
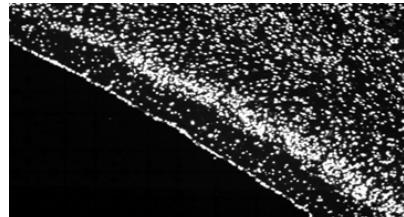
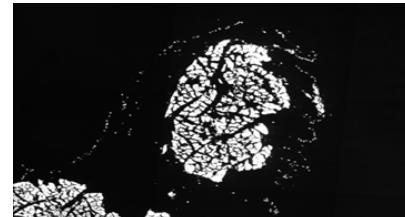
⁴The presence of “ghost” tails at the cells will affect the downstream cell segmentation analysis. Violent sliding of cover slip over the tissue which burst the cells, or vigorous air blowing on the tissue by a power dust-remover can be the cause of this occurrence.



Imaging factors that will influence cell segmentation and cellbin results:

- ① Capture images which cell boundaries could be clearly identified with naked eyes.
- ② Image blur score should be above 80.
- ③ Image MUST pass QC.
- ④ Make sure that the stitching error is < 5 pixel (Contact and make an appointment with the microscope engineer of the microscope brand you are using for assistance if your image shown stitching error > 5 pixel)
- ⑤ Tissue type and cell density will affect final cell segmentation in different aspects

Cell segmentation and cellbin results will be greatly compromised if captured images fail to meet no. 1-4.

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

CHAPTER 3

MICROSCOPE ASSESSMENT

3.1. Imaging System Requirements

In general, a fluorescent imaging system being used should:

- a. Be able to perform tile scanning to image a minimal area of 10mm x 10mm (standard STOmics chip size).
- b. Have image stitching function either via the microscope's software or equivalent such as ImageJ.
- c. Have an image resolution ≥ 1800 pixels (height) and ≥ 2000 pixels (width).
- d. Be able to view and save stitched image in raw file format (e.g., czi in Zeiss) and export it into TIFF/ PNG.
- e. (Optional): be able to view and perform automatic or manual saving of tile images (individual FOV) in both raw file and TIFF/ PNG formats (required for API development).

Additionally, the computer connected to the microscope should also be able to handle large images (>5 GB) and allow installation of a third-party image processing software such as ImageQC (section 4.1).

If none of the microscopes recommended in Table 1 (section 2.1) is available, the prospective microscope should also have **ALL** the properties and functions listed in Table 3.

Table 3 Overview of imaging system requirements

Index/ Parameter	Description
XY stage travel distance of microscope	At least 25*75mm
Microscope type	Upright microscope
Focusing approach	Pre-focus map, Real-time autofocus
Fluorescent channel	DAPI, FITC, TRITC, CY5
Objective lens	4X, 10X (NA ≥ 0.3)
Camera resolution	Camera resolution selection depends on objective lens. Please consult your microscope vendor for details
Image bit depth	16 bits
Background balance	Adjustable background balancing function
Distortion correction	Adjustable distortion correction function
Overlap ratio	Adjustable, 10% by default
File format	Is capable of viewing and exporting stitched image, 8bit/16bit, TIFF/ PNG, grayscale or colored images
PC requirement	Windows 10 x 64 system, 16G memory or beyond

3.2. Pre-assessment Preparation

Materials

- Slide dryer set to 50°C
- Stereo-seq Microscope Assessment Chip T (1cm*1cm) (STOmics, Cat. No. 101CT111)
- Nuclease-free water (NF water; Ambion, Cat. No. AM9937)
- Staining reagent: Qubit® ssDNA Assay Kit (Invitrogen, Cat. No. Q10212)
- 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.

3.3. Microscope Assessment Procedure

1. Prior to microscope assessment:

Please contact and make an appointment with the microscope engineer of the microscope brand you are

using. After imaging with the test chip and passing QC, confirm the information of all parameters and do not change them (such as FOV height, FOV width and image ratio information) before the official experiment.

2. During microscope assessment:

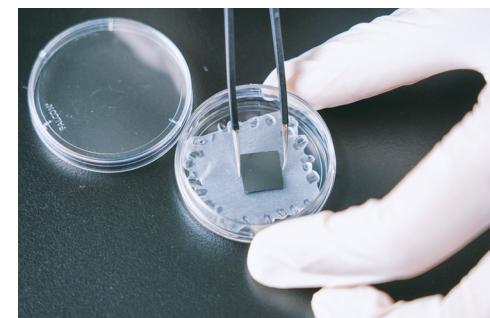
Chip staining

- 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 dusts 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 at 50°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.



- d. Add staining solution prepared according to Table 4 onto the front-side of the chip, followed by an incubation of 5 minutes in the dark (covered with a paper box or aluminum foil).

Table 4 Stereo-seq Microscope Assessment Chip staining solution

Component	Volume
Invitrogen® Qubit ssDNA Buffer	99.5 µL
Qubit ssDNA Reagent	0.5 µL
Total	100 µL

- e. After that, remove the staining solution from the chip corner with a pipette. Rinse the chip with 100 µL of nuclease-free water. Dry the surface of the chip with a dust remover. Then, place the chip into a new petri dish and cover it with aluminum foil or paper box to keep it in the dark before imaging.



Please make sure to remove as much staining solution as possible and clean the chip with 0.1x SSC to prevent excessive salt stains on the chip surface affecting the imaging quality. Make sure to completely dry the 0.1x SSC solution as well using a dust-free paper to first absorb the solution from the side of the chip, and then gently blow dry the residual liquid with a power dust remover.

Fluorescent Imaging

- a. Place the chip directly on the imaging stage of a microscope or attach it onto a glass slide before placing onto the stage. For better adherence, simply add a drop of water (1 µL) onto the slide before placing the chip on it. Mind the angle of the chip on the imaging stage, it should not be tilted.

- b. Use FITC channel and 10x objective lens to capture image of the entire test chip including its four corners using the fluorescence channel.
 c. Save the stitched image in both raw file format and TIFF/ PNG formats. Record image parameters (refer to section 4.2) and proceed to image evaluation.

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

Evaluation of fluorescent images via ImageQC software

- a. Download and install the latest version of ImageQC software. Download links are provided in section 4.1. For installation guidelines, refer to section 4.2.
- b. Follow instructions provided in section 4.3 to perform image evaluation via ImageQC software. Different input image files and formats are required for the software, depending on the used microscope, kindly refer to Table 2 in section 2.3.

CHAPTER 4

ImageQC SOFTWARE

4.1. General Information

STOmics microscope ImageQC software is intended for assessing the quality of microscope images. Besides resolution and sharpness, it also evaluates stitching quality of a Stereo-seq chip image by referring to the background tracklines. The ImageQC software has two main applications. Firstly, a fluorescent image of a tissue mounted Chip T (STOmics, Cat. No.: 110CT114) should pass the ImageQC evaluation to ensure that it fulfills requirements for the upstream data analysis via SAW pipelines. Secondly, with the aid of a Stereo-seq Microscope Assessment Chip T (STOmics, Cat. No.: 101CT111), the software can also be used to assess if a microscope is compatible with the STOmics protocols.

Input and Output files of ImageQC

- Different input files in their corresponding formats are used for ImageQC software based on the microscope used. They are listed in Table 2 of section 2.3.
- Two output files are generated after an image has passed the evaluation – json file and tar.gz compressed file. These ImageQC output files can be found in “QCImgUpload” folder and will also be uploaded to a corresponding computer cluster or cloud and used for data analysis via SAW pipelines. To set up SAW pipelines in a computer cluster or cloud, please contact a BGI FAS, who will connect you with our IT team.

This PC > Recovery (D:) > AutoUpload > QCImgUpload			
	Name	Date	Type
> Quick access	SS200000116TL_F3_20220921_205008_1.1.3.ipr	9/21/2022 8:51 PM	IntelliJ IDEA Project File
> OneDrive - BGI Tech S	SS200000116TL_F3_20220921_205008_1.1.3.tar.gz	9/21/2022 8:52 PM	GZ 压缩文件

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 (refer to section 4.2, step j. for details).

ImageQC software Download links

Github: <https://github.com/BGIResearch/imageQC>

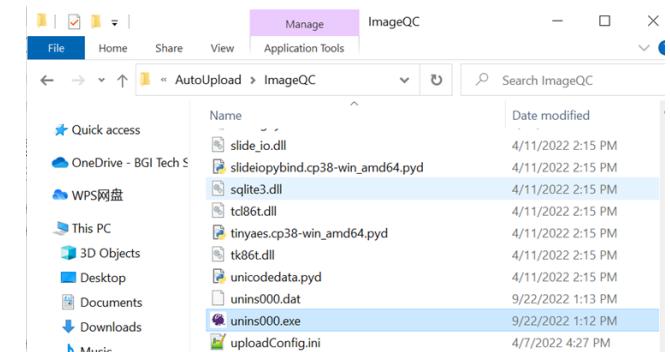
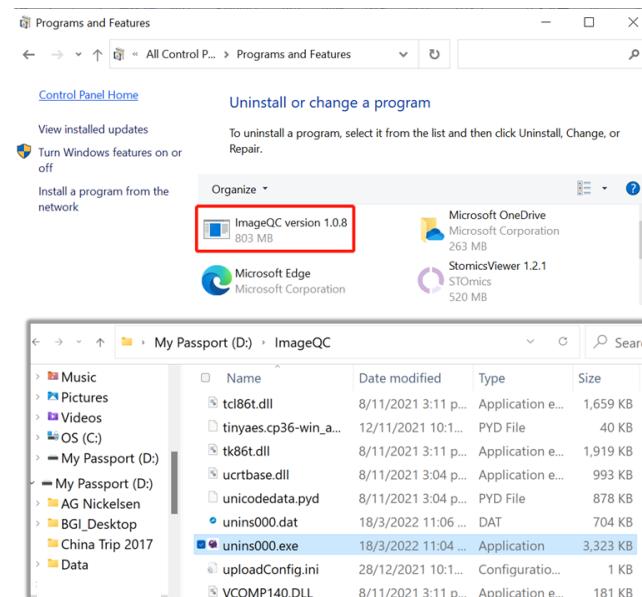
Or https://drive.google.com/file/d/1vT3oHsSlvieg84jliu_dLzYWlqmNyB0/view?usp=sharing

4.2. Software Installation

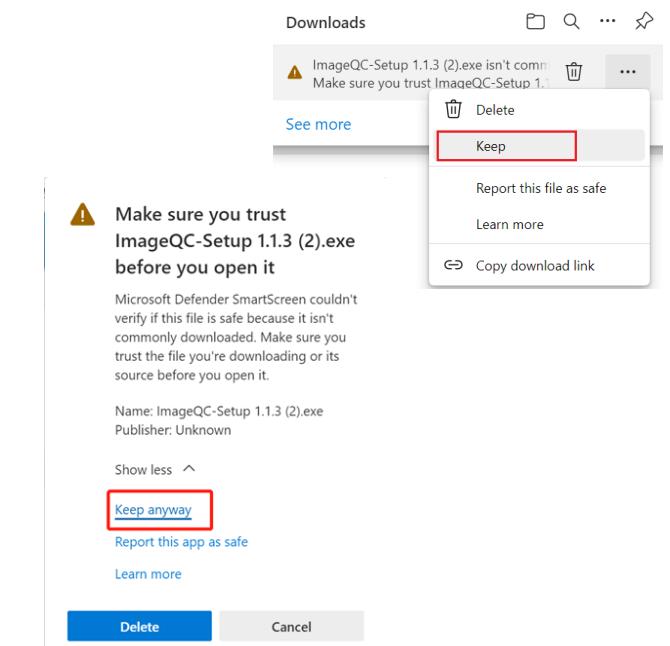
a. If ImageQC v1.0.8 or an earlier version has been downloaded previously, please refer to step b. Otherwise, skip step b. and proceed to step c.

b. Please uninstall an earlier ImageQC version from the control panel or delete “unis000.exe” file from the same folder in which the ImageQC program is located, as shown in the two screenshots below:

 When updating the software, you may choose NOT to delete the folder “QClmgUpload” where all previous image evaluation result files and information directories are saved.



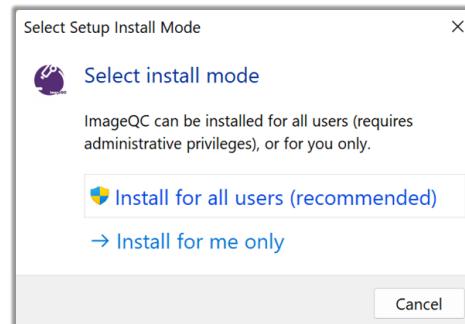
c. Download “ImageQC-Setup v1.1.3.exe” installation package from links given in section 4.1. The software installation might get blocked, simply click on the “...” button and “Keep” to recognize the software installation package as a trusted file.



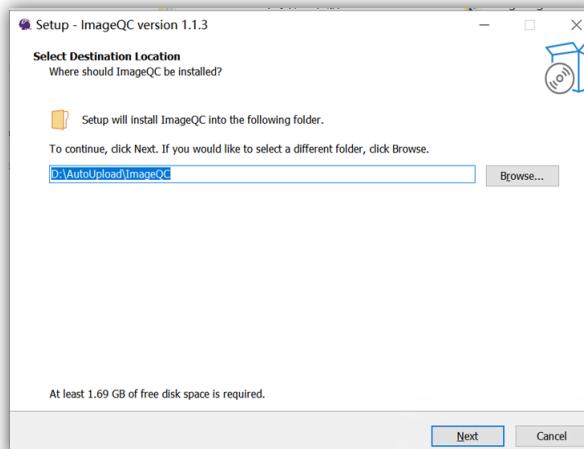
- d. Double click the downloaded file “ImageQC-Setup v1.1.3.exe”



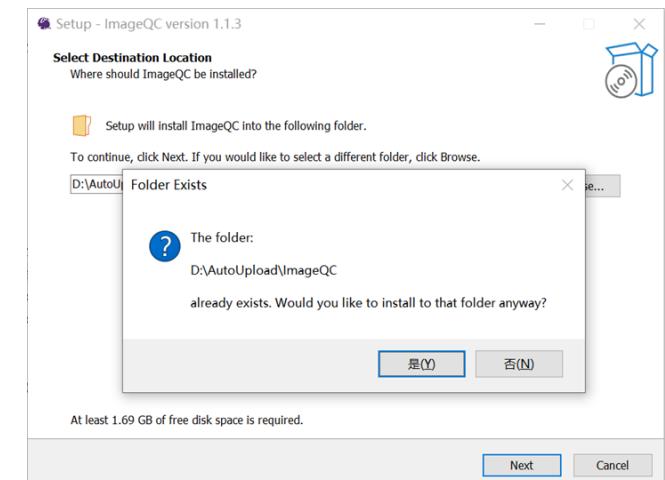
- e. Select an install mode. “Install for all users” mode is recommended.



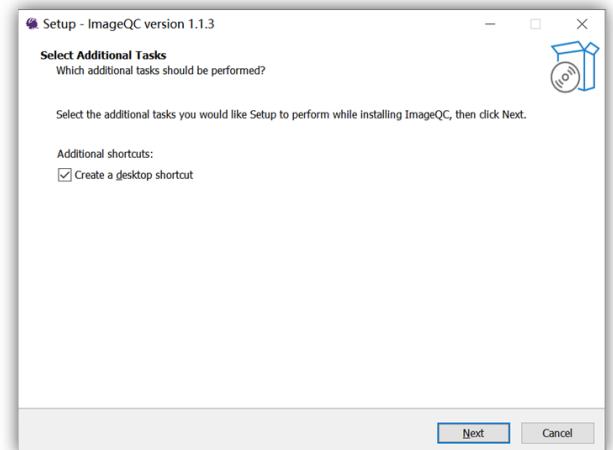
- f. Select a destination location. Installation into the C: Drive may require administrative permission. Hence, you are advised to install it into the D: Drive.

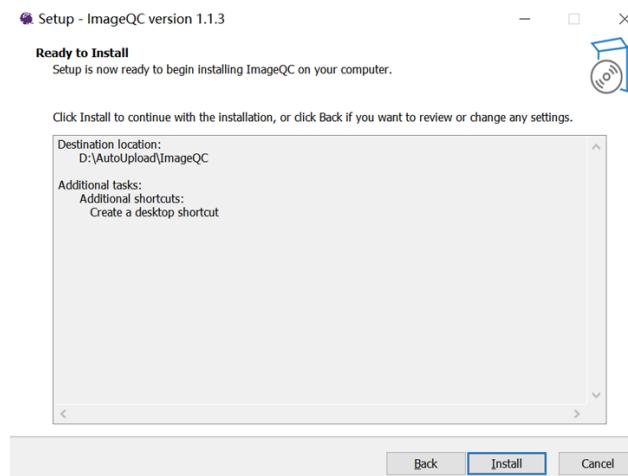


- g. If an earlier version of ImageQC software has been installed before, select “yes” in the pop-up window shown below:

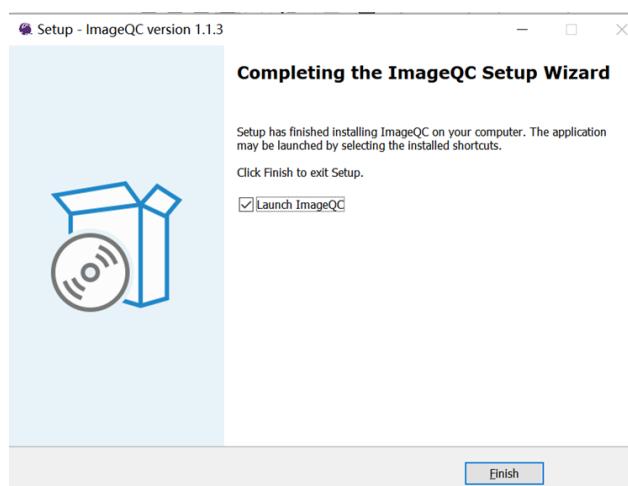


- h. Select if a shortcut should be created and click “install” to start the software installation.





- i. After installation is completed, tick the option “Launch ImageQC” and click “Finish” to launch the program.



Once the installation is completed, there will be an “**ImageQC**” folder generated in the installation destination chosen in step g. Another folder – “**QCImgUpload**” will appear immediately upon the first run of image evaluation by the program.

My Passport (D:) > AutoUpload			
	Name	Date modified	Type
>	Desktop	8/6/2022 4:36 pm	File folder
>	Documents	8/6/2022 4:20 pm	File folder
>	Downloads		
	ImageQC		
	QCImgUpload		

- j. If this is the first installation or an update from v1.0.8 or earlier version, please check if there is a file named “settings.json” in “ImageQC” folder. If there is not, please open the newly installed ImageQC program, the file will be auto generated immediately.

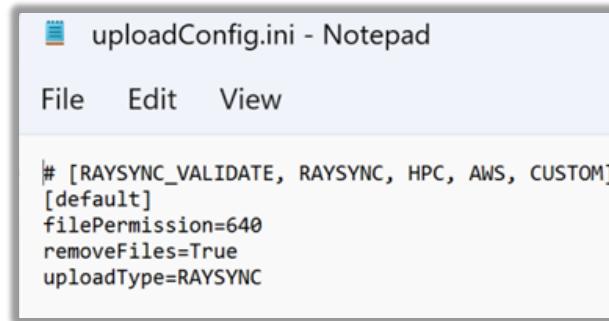
My Passport (D:) > AutoUpload > ImageQC			
	Name	Date modified	Type
>	Desktop	11/4/2022 2:15 pm	PYD File
>	Documents	22/4/2022 8:25 pm	JSON File
>	Downloads	11/4/2022 2:15 pm	Application extens
>	Music		
	select.pyd		
	settings.json		
	slide_io.dll		

The “setting.json” file is a configuration file for ImageQC output file uploading to a corresponding computer cluster or cloud, upon evaluation. Open this “setting.json” file with Notepad application; there are 4 clauses in the file, edit them if necessary:

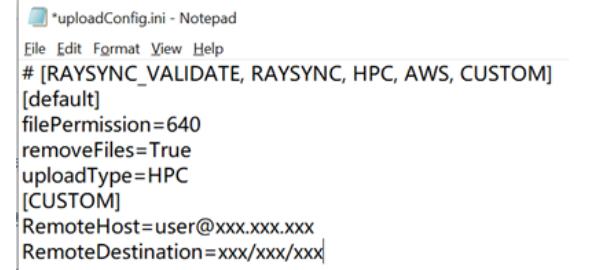
```
settings - Notepad
File Edit View
{
  "qc_flag": true,
  "upload_flag": true,
  "language": "eng",
  "cut_czi": false
}
```

- **qc_flag:** Clause for the operation of image evaluations. Do not make any changes.
- **upload_flag:** Clause that control ImageQC output file auto-uploading. “True” is set as the default, implying an image is going to be uploaded automatically once it has passed the evaluation. If auto-uploading is not required, change it into “false”.
- **language:** Clause for the program interface language. The default is “chn” which stands for Chinese. If an English interface is preferred, please change it to “eng”.
- **cut czi:** Clause controlling czi file processing. Do not make any changes.

k. Please also open with Notepad application “uploadConfig.ini” file which can be found in “ImageQC” folder, and check the image uploading method:



```
# [RAYSYNC_VALIDATE, RAYSYNC, HPC, AWS, CUSTOM]
[default]
filePermission=640
removeFiles=True
uploadType=RAYSYNC
```

```
File Edit Format View Help
# [RAYSYNC_VALIDATE, RAYSYNC, HPC, AWS, CUSTOM]
[default]
filePermission=640
removeFiles=True
uploadType=HPC
[CUSTOM]
RemoteHost=user@xxx.xxx.xxx
RemoteDestination=xxx/xxx/xxx
```

- **filePermission:** Permission to access ImageQC output files uploading
- **removeFiles:** Deletion of intermediate files generated in the local system during image evaluation
- **uploadType:** (There are four clauses)
 - 1) HPC is for local upload of ImageQC output files to the computer cluster in Shenzhen. Please set up the network configuration in advance, BGI intranet is required.
 - 2) RAYSYNC and RAYSYNC_VALIDATE, both uploads ImageQC output files via Raysync software to the Shenzhen’s computer cluster. It requires only internet, and no additional network configuration. RAYSYNC_VALIDATE is for external ImageQC users, the parameter will be changed to RAYSYNC automatically by the program once an image has passed the evaluation and the output files have been uploaded successfully.
 - 3) AWS stands for Amazon Web Service. ImageQC output files will be uploaded to Amazon cloud. AWS account password and Python language installation are required. Later versions of ImageQC software will have AWS path configuration function.
 - 4) CUSTOM stands for customized RSYNC upload account and path, applicable to areas where BGI’s proprietary Stereo-seq Analysis Platform is deployed (only in selected regions). After QC, the data is uploaded to its own cluster for analysis.

4.3. Software Operation Instructions

Follow the procedure below to run an image evaluation via ImageQC:

- Click and open “ImageQC.exe” in “ImageQC” folder to run the ImageQC program after imaging.
- Drag and drop image files or folders into the program window.
 - Zeiss Axio Scan Z1 or Axio Scan 7: drag and drop a raw image file in czi format.
 - Leica DM6-M: drag and drop a stitched image in TIFF/ PNG format
 - Other microscopes (before API development): drag and drop a stitched image in TIFF/ PNG format
 - Other microscopes (after API development):
 - enter the path where exported tile images (individual FOV) in TIFF/ PNG format are saved.
 - drag and drop a raw image file in the original format generated by the microscope software.

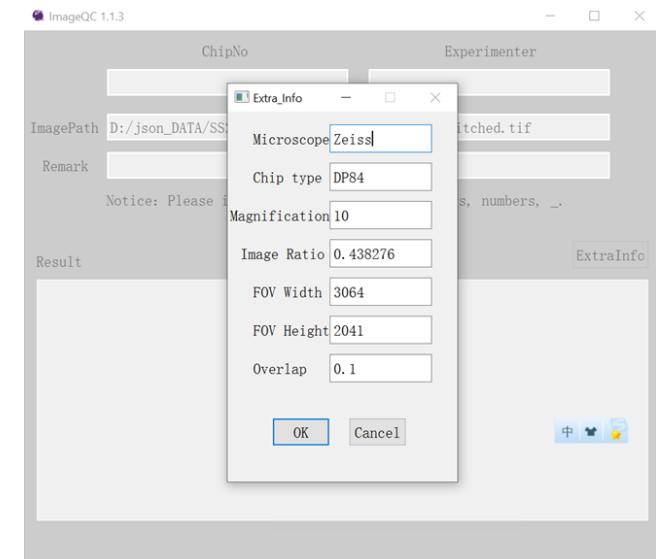


The required input files and format depend on the API development method if the microscope. Please consult the corresponding BGI FAS.

- Users will be requested to fill in image parameters manually in the pop-up window shown in the screenshot below:

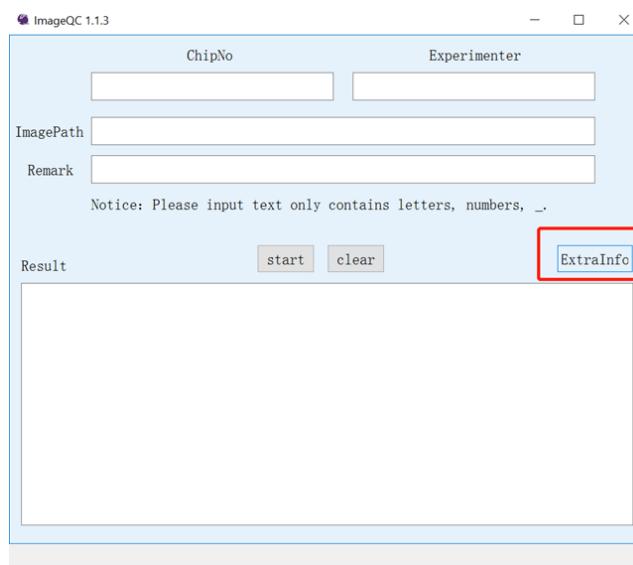


These parameters are not required if exported tile images in TIFF/ PNG format are used.

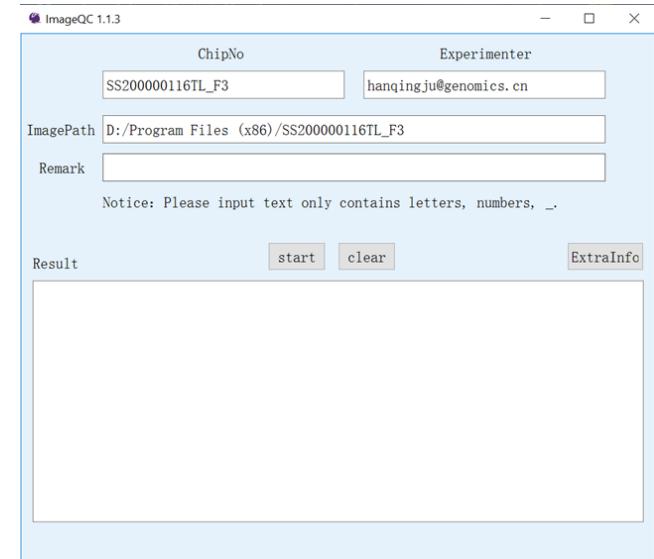


- Microscope:** Brand name of the microscope such as Zeiss, Leica, Motic, Olympus, etc.
- Chip type:** It is the first 3 to 4 letters or numbers of a chip number. For examples, FP2, SS2, DP8, DP84.
- Magnification:** Image magnification. It should be 10, if a 10x lens was used to take the image.
- Image ratio:** Image ratio can also be defined as 1/ resolution, and hence can derive from the image resolution. It should be in the unit of $\mu\text{m}/\text{pixel}$, with at least 3 decimal places.
- FOV width and FOV height:** Width and height of a tile image or individual FOV (in pixel)
- Overlap:** Overlap ratio between two adjacent FOVs (if the overlap ratios at the width and height are different, take the bigger value). The value should be 0.1, if there is a 10% overlap.

The program will memorize the image parameters and fill them in automatically in the next visit. Overwrite them if needed. If any of these details is wrong or missing, especially when the input file is a stitched image, the program will not be able to perform an evaluation properly. For parameter editing, click “**ExtraInfo**” at the bottom right corner as illustrated in the screenshot below:



d. Key in the chip number and the operator's email address.



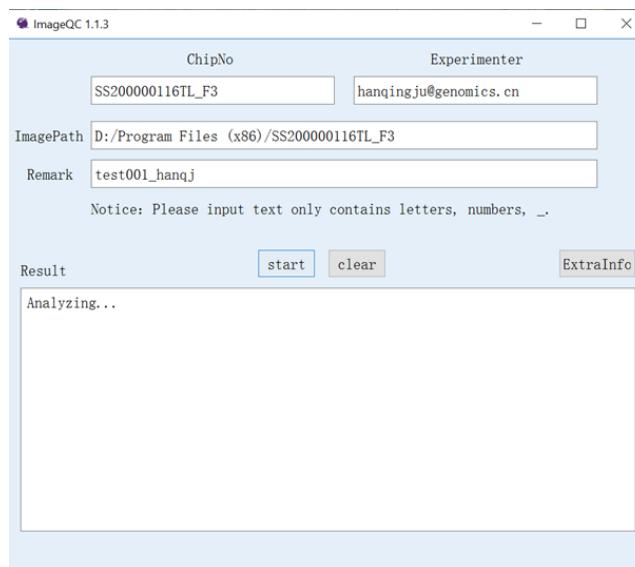
- **Chip Number:** Chip number of a Stereo-seq Microscope Assessment Chip or Chip T used in imaging. ImageQC software only recognize a chip number of BGI STOomics.

- **Experimenter:** Full email address of the operator

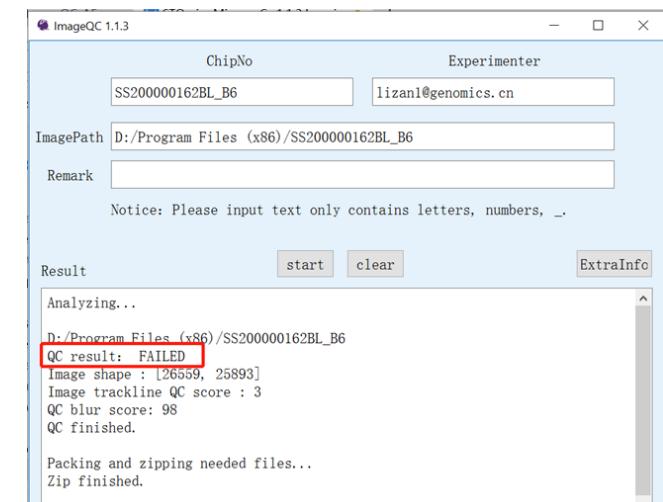
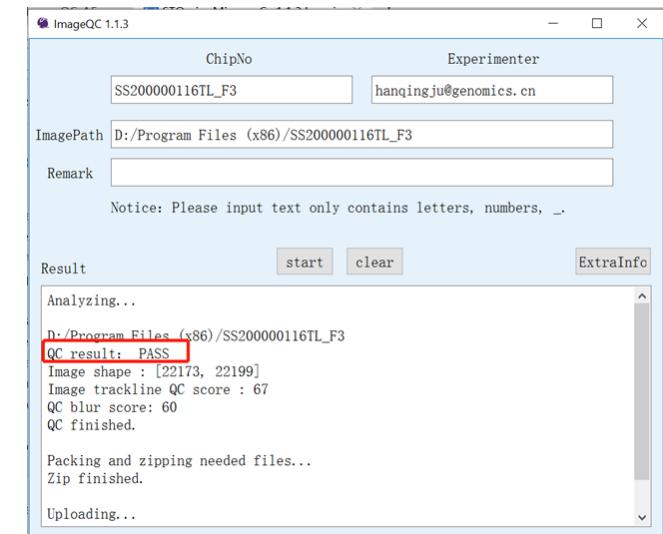
The chip number and the experimenter's email will be recorded and filled automatically in the next visit. For a new user, please type in new information to overwrite the previous ones.

- **Remark:** Record additional details in the remark field if needed.

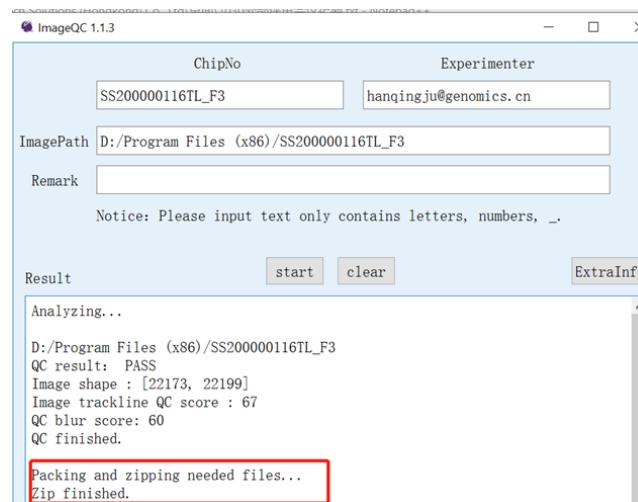
e. Click the "Start" button to start the evaluation



f. When an evaluation is completed, it will be indicated as "QC finished" in the last line of the window. The program will also display the evaluation result as either "PASS" or "FAILED". If the latter result is obtained, please check if your image was taken by following the imaging guidelines instructed in Chapter 2. The user may also consider retaking a new image. Contact a STOmics FAS for assistance if necessary.

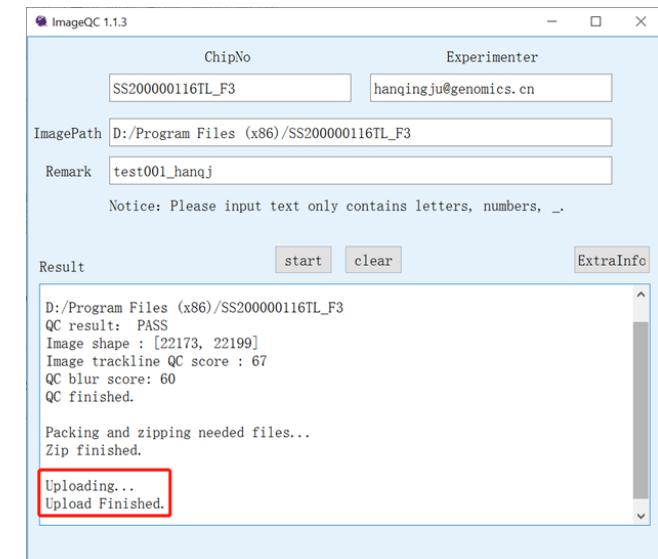


g. Once an image has passed the evaluation, the program will compress ImageQC output files (json and tar.gz files) as demonstrated in the screenshot below:



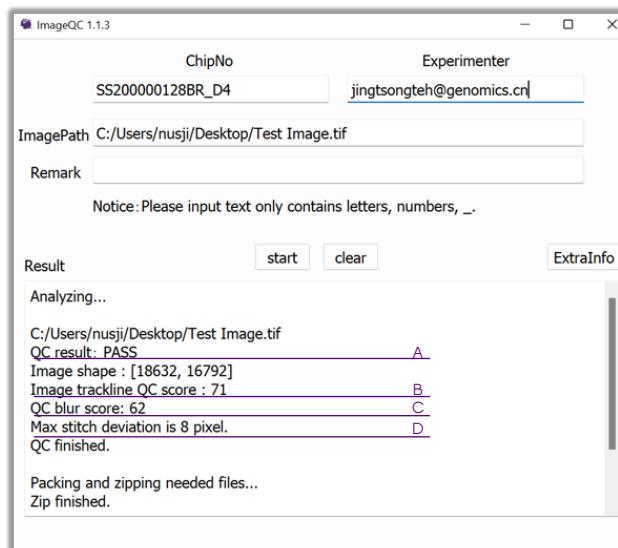
h. The ImageQC output files will then be automatically uploaded (if auto-uploading function is preset in section 4.2, step 10) to a corresponding computer cluster or cloud as shown in the screenshot below:

If data upload is not required, click "clear" to discard contents in the program window. The program is then ready for a new image evaluation.



4.4. Interpretation of ImageQC Evaluation Results

An image evaluation result of the ImageQC software consists of a few components. As shown in the diagram below, they are QC result, Image trackline QC score, QC blur score and Max stitch deviation.



A. QC result:

The QC result is the overall result of the evaluation. In the case of tissue mounted Chip T image, it indicates if the image is qualified to be used as a map on which the transcriptomics data are overlayed and visualized. On the other hand, in the evaluation of a microscope assessment chip image, it tells if a microscope is compatible to the STOmics protocols. There are two possible outcomes: PASS or FAILED. 'PASS' indicates a qualified image and a compatible microscope, while 'FAILED' means the opposite.

B. Image trackline QC score:

Tracklines on a Stereo-seq Chip T is important for calibration and registration of a tissue stitched image to the transcriptomic data. Hence, when performing tissue mounting onto a Stereo-seq Chip T, users should ensure that at least 20% of the chip area remains uncovered by the tissue.

Tile images which constitute a stitched image can be classified based on the number of tracklines that are clear and detectable by the ImageQC software.

- 1. Model tile image:** a tile image with at least three vertical and three horizontal tracklines detected.
- 2. Qualified tile image:** a tile image with at least one vertical and one horizontal tracklines detected.
- 3. Unqualified tile image:** a tile image which does not fulfil the criterion 2 stated above.

As mentioned before, to pass the evaluation, a stitched image of a microscope assessment chip or a tissue mounted Chip T should have at least one model tile image and hence has an image trackline QC score of 60 and above, out of 100, as shown in Table 5. Further, the trackline QC score of a microscope assessment chip image is usually higher than that of a Chip T image because most of the tracklines on the Chip T are covered by the tissue.

Table 5 Derivation of different Image trackline QC scores.

Image trackline QC score	Criteria	ImageQC Result
< 60	No detection of any model tile image	Fail
= 60	Detected one model tile image	Pass
> 60	<p>Detected at least one model tile image and one or more qualified tile images</p> $P = \frac{\text{Number of qualified tile images}}{\text{Total number of tile images}}$ <p>Score = $60 + f(x) =$</p> $\begin{cases} p * \frac{20}{0.82}, & 0 < p \leq 0.82 \\ 20 + (p - 0.82) * \frac{20}{0.82}, & p > 0.82 \end{cases}$	Pass

If there are three vertical and three horizontal tracklines can be clearly seen with naked eyes on a tile image, yet the image failed in the evaluation, please feedback to local STOmics FAS. They could perform a more thorough evaluation for the image.

C. QC blur score:

The evaluation is performed by a deep learning model based on convolutional neural network, developed by BGI Research. The score presents the blur and sharpness of a tissue image on a Chip T. Distinct boundaries between cells (nuclei) are a key criterion to perform cell segmentation analysis, and hence highly depending on tissue type and its cell density.

The QC blur score will not affect the final result of the evaluation – QC result, it only serves as a reference to assess the feasibility of performing single cell segmentation based on the nuclear stained image. The score has a scale of 0 – 100, a higher score represents a better tissue image quality, and a minimal score of 80 is required for cell segmentation. Take note that a score of zero is obtained for a microscope assessment chip image which contains no mounted tissue.

Averaged Template FOV Score	Image Blur Score
0~0.2	0~60
0.2~0.8	60~80
0.8~1.0	80~100

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

D. Max stitch deviation:

Stitching accuracy is another key requirement for cell segmentation analysis. Image with huge stitching errors (error greater than 5 pixels) will compromise the cell segmentation results. Max stitch deviation reflects the biggest stitching error detected on a tissue image.

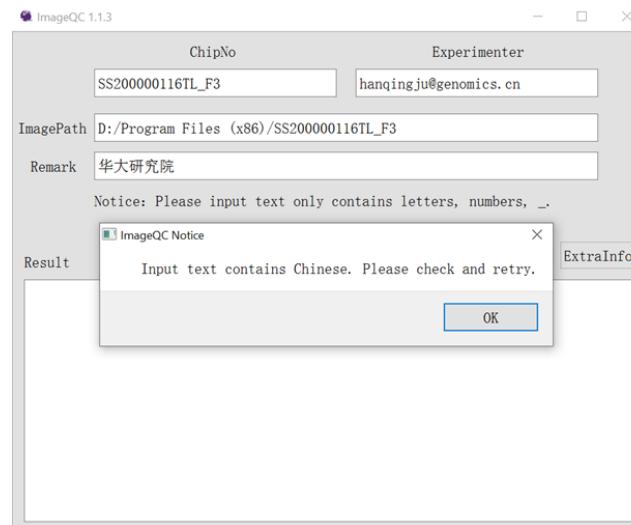
What to do when QC failed

- If your Image didn't pass QC, there is no valid image data to run the `register` and `cellCut` pipelines in SAW so that tissue and cell segmentation cannot be performed based on your ssDNA image. In this scenario, SAW can still identify and extract tissue regions based on the gene expression matrix by running the `tissueCut` pipeline, and output the standard analysis report.
- If the QC failure is caused by the misjudgment of the ImageQC software algorithm, i.e. QC failed when three horizontal and vertical tracklines are visible to the naked eye, please contact your local STOmics FAS in time.

- Images could fail ImageQC due to differences in microscope parameters. If after multiple try outs the image still failed, please contact the microscope engineer to help troubleshoot whether it could be fixed by re-adjusting the imaging parameters, etc.
- If your Image is not passed due to unfamiliarity of the experimenter with the microscope or ImageQC software, please contact your local FAS in time.

4.5. Errors & Troubleshooting

- a. If there is a Chinese character in the input text, an error message will appear as shown below:



- b. If the chip number is missing or an unrecognized chip type has been entered.

a.

Please make sure that the input content and image path do not contain any Chinese character. Remove the character to proceed.

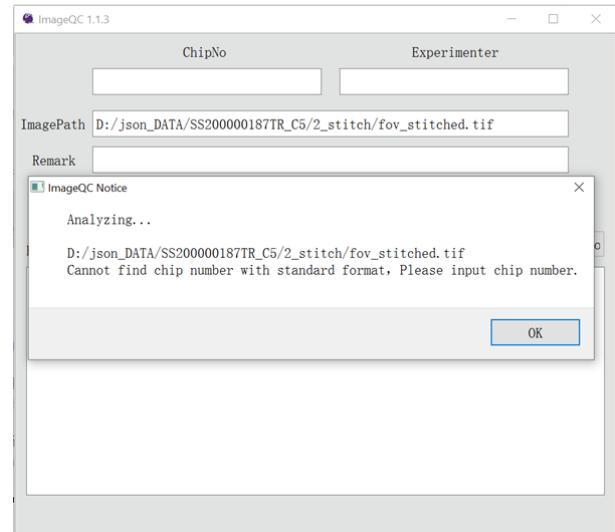
2)

The last line shows chip types supported by ImageQC software.

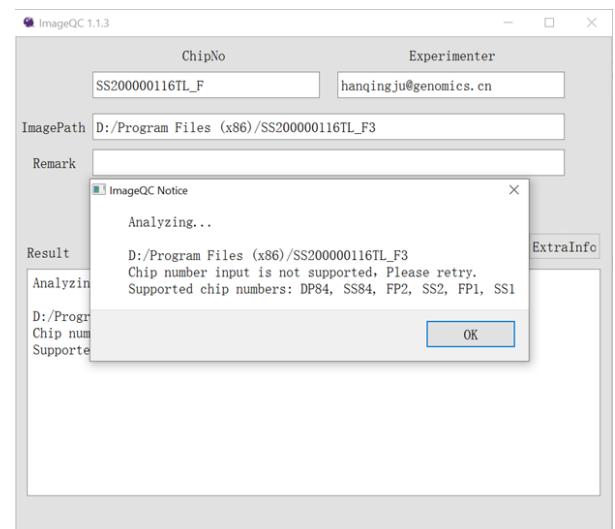
Fill in or correct the chip number and resume the evaluation.



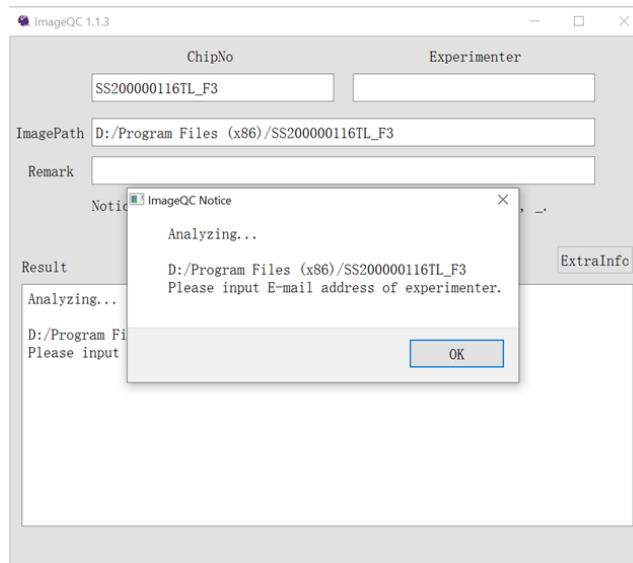
- 1) If the chip number is missing, the following window will pop up:



- 2) If a wrong chip number is entered, the following window will appear:

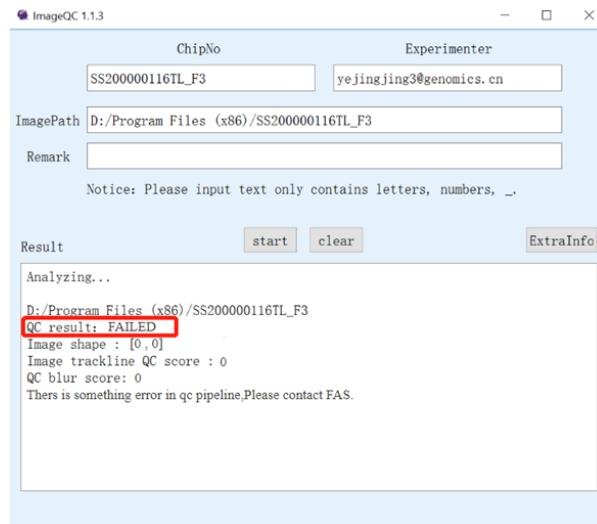


c. If the experimenter's email address is missing, the following window will appear:



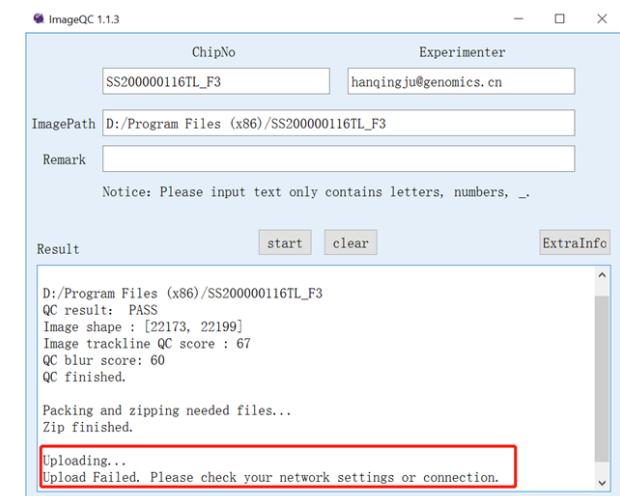
Fill in the email address and resume the evaluation.

d. If it is indicated in the QC result that the image shape is [0 0] as shown in the following:



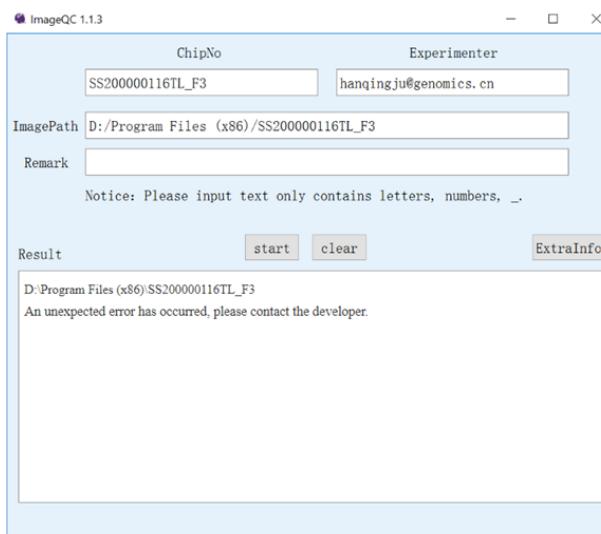
This is probably due to the program's internal errors. Make a screenshot of the window and contact a STOmics FAS for assistance.

e. When failed to upload the ImageQC output files, the following message will show:

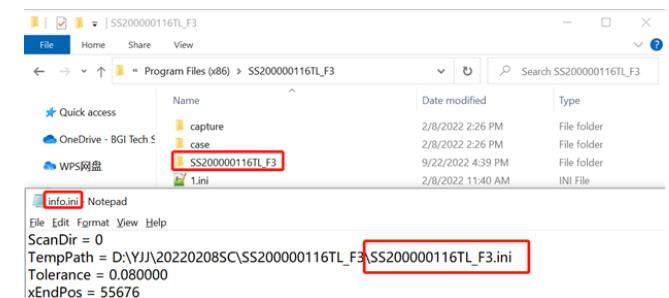


Please check the internet connection to resume the evaluation.

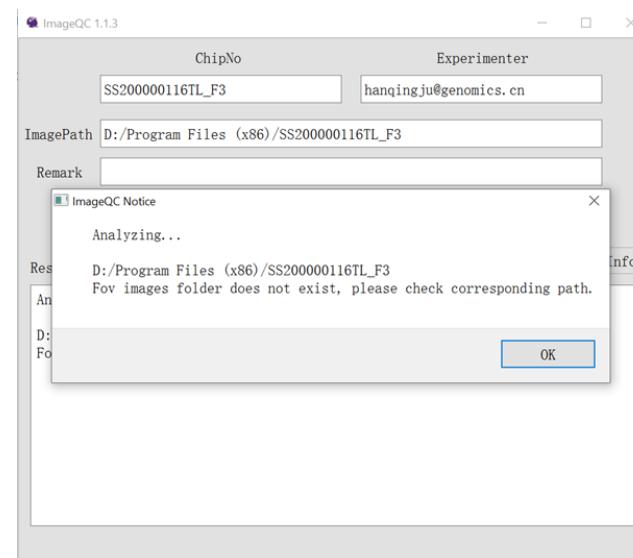
f. If an unexpected error occurs, the program will display the following content:



 Please check all image parameters entered. If the issue is still not resolved, please send the image and the corresponding parameters to a FAS of BGI, they will provide feedback to the software developers.



g. FOV image folder non-existing



 Please check if there a FOV image folder has been generated for your chip. If there is one, open the info.ini file and find TempPath. Change the file name of the path to 'tile image folder name + .ini'.