USER MANUAL

RNAscope™ HiPlex Image Registration Software v2.0.1 User Manual

Document Number 300065-UM

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1 Chapter 1. Product Information

IMPORTANT! We recommend reading the entire user manual before using the software.

About this guide

This user manual provides guidelines and steps for using the RNAscope HiPlex Image Registration Software (Cat. No. 300065). Refer to the RNAscope HiPlex12 Reagents Kit (488, 550, 650, 750) v2 Assay User Manual (Doc. No. 324400 UM) or RNAscope HiPlex12 Reagents Kit (488, 550, 650) v2 Assay User Manual (Doc. No. 324410 UM) - to detect target RNAs and image your tissue samples.
Visit www.acdbio.com/technical-support/user-manuals to download user manuals.

Product description

Background

You can use the RNAscope HiPlex Image Registration Software to register and merge the images you have acquired after performing the RNAscope HiPlex Assay. The assay is based on ACD's patented signal amplification and background suppression technology and incorporates multiplexed signal amplification systems, enabling users to investigate expression as well as positional relationship between multiple genes within a cellular context.

Overview

The RNAscope HiPlex assay uses cleavable fluorophores to detect up to four targets at a time. To detect all targets, multiple rounds of target detection and imaging with an epifluorescence microscope with the appropriate filters are performed. For a more in-depth overview of the RNAscope HiPlex Assay, refer to the RNAscope HiPlex12 Reagents Kit (488, 550, 650, 750) v2 Assay User Manual (Doc. No. 324400 UM) or RNAscope HiPlex12 Reagents Kit (488, 550, 650) v2 Assay User Manual (Doc. No. 324410 UM).

After you have acquired your images, use the RNAscope HiPlex Image Registration Software to merge/register the images together. Because you are performing multiple rounds of detection and imaging, we recommend including initials such as R1 (round 1), R2 (round 2), R3 (round 3) and so on, as well as the target names when saving image files. Implementing a naming convention will help identify each group of images during the image registration process. The registration process involves

generating transformation matrix files (*.tfm) using single DAPI channel images to serve as references to register the rest of the single channel images. You will either generate one or multiple files depending on the number of detection rounds performed and the number of DAPI images acquired. The first DAPI image opened in the software will serve as a "default" reference image for the second DAPI image, generating a single transformation matrix file. If you have completed a third round of detection, a second file is generated when the third DAPI image is aligned with respect to the first DAPI image. Once the transformation matrix files are generated, apply them to your single channel images. The *.tfm files will not be applied to any of the reference images. If your images are acquired with an FFPE sample or other type of samples with high autofluorescence, you also have the option to reduce background in the software. Once all of the images have been merged, you can analyze the composite image and investigate the expression of the target RNAs.

If you have any questions, contact technical support at support.acd@bio-techne.com.

Software license

The RNAscope HiPlex Image Registration Software is available by download from the link obtained from ACD.

2 Chapter 2. Before You Begin

Before using the software to merge your images, keep the following considerations in mind.

Important considerations

- After you have acquired your images, name your files carefully. As you will be performing multiple rounds of detection and imaging, we recommend including initials such as R1 (round 1), R2 (round 2), R3 (round 3), etc. and the target names when saving image files. Implementing a naming convention will help identify each group of images during the image registration process.
- Make sure that the input images are single channel and in the "Tag Image File Format (tiff)" file format (gray scale or pseudo colored). You should have up to five images per round (DAPI, AF488, DL550. DL650, AF750). Make sure that single channel images do not have DAPI overlaid.
- All input images should have the same resolution and pixel by pixel dimensions. The RNAscope HiPlex image registration software cannot register a cropped image to an uncropped image. Make sure that images do not have a scale bar.
- RNAscope HiPlex Image Registration software can handle images of any size, but larger images take longer to process and view. To achieve faster performance, we recommend processing a field of view of no more than 1024 x1024 pixels.
- For best results, we recommend having an overlap of about 70% between your images.
- For best registration accuracy, make sure that the DAPI channel images are similarly exposed as the other images (the intensity profile of the nuclei is similar). You may have to adjust the exposure to achieve similarly exposed images. Overexposure could affect the nuclear size and shape, resulting in registration failure due to a mismatch of the nuclear boundary.
- The registration process involves generating transformation matrix files (*.tfm) using single channel DAPI images that serve as references to register the rest of the single channel images.
- The first DAPI image that you open in the software serves as a "default" reference image for the DAPI from all other rounds.
- You will either generate one or multiple *tfm files depending on the number of detection rounds performed and the number of DAPI images acquired. The *tfm file is a data file that is used to align all the other channels since the data points in a single round are already aligned to DAPI staining.
- Once the transformation matrix files are generated, apply them to your single channel images. The *.tfm files are not applied to any of the reference images from Round1. Once all of the images have been merged, you can analyze the composite image and expression of the target RNAs.

- The Remove Background without a Blank Image function (a blank image is an image acquired without probe signals, either before probing or after probe cleavage) provided in this software can cause false positives and false negatives while detecting probe dots. The function can have difficulties distinguishing large clusters of dots from autofluorescence, as the clusters can resemble the pattern of autofluorescence in certain tissue types. In addition, dot-like or filamentous autofluorescence patterns can be accidentally recognized as probe dots. Exercise caution when processing images with this function.
- The Remove Background with a Blank Image function can also cause false positives and false negatives while detecting probe dots. Exercise caution when using this function, especially when adjusting the parameters. Non-probe objects, if they are only seen in the probe images, can still remain after applying this function. We recommend starting with the default parameter values and referring to this user manual as a guide (see the section *Remove background (optional)* in Chapter 4).

3 Image Registration Software Menu

This chapter describes the basic functions available in the RNAscope HiPlex Image Registration Software.

File menu

The following options are available under the File menu.

Open

Use this option to browse to the source folder containing your image files. If any files are already open in the software, this option overwrites the current list.

Add Images to Current List

Use this option to add additional files to the current list of open images. The new files are added to the end of the list.

Save Images

Select one of the following options under the Save Images menu:

- Choose Save All Images (Gray) to save each image separately as a single-channel, gray scale image.
- Choose Save All Images (Color) to save each image separately as a single-channel, colored image. The saved images will include the display adjustments (pseudo-color, Brightness, Contrast: Min, Contrast: Max, and Threshold), but will not include any zoom adjustments.
- Choose Save Checked Images to save the images you have checked as single-channel, gray scale images.
- Choose Save Composite Image to save the current displayed image as a single, composite RGB image. The saved image will include the display adjustments (pseudo-color, Brightness, Contrast: Min, Contrast: Max, and Threshold) and the zoom adjustment.
- Choose Save Omero Tif to save all images as a multi-channel, gray scale image.

Exit

Use this option to quit the software without saving any information.

Edit menu

The following options are available under the Edit menu.

Select All

Use this option to select all of the images that are open in the RNAscope HiPlex Image Registration software.

Unselect All

Use this option to deselect all of the images in the list of open files.

Remove Selected Images

Use this option to remove the selected images from the list of open files.

Register menu

The following options are available under the Register menu.

Register DAPI Images

Use this function to register selected DAPI images and write the transformation matrix. This is the essential first step for generating a multiplex image.

Apply Registration Transform

Use this function to apply your generated registration transformation matrix on selected single-channel images.

Crop Overlapping Region

Use this option after you have registered all single-channel images to crop the overlapping region of all of the images in the list. No image selection is needed.

Preprocess menu

The following options are available under the Preprocess menu.

Remove Background without a Blank Image

Use this function to reduce the autofluorescence background in the selected images. Use this function when a blank image is not available. A blank image is an image acquired without probe signals, either before probing or after probe cleavage.

Remove Background with a Blank Image

Use this function to reduce autofluorescence background in the selected images by subtracting a blank (autofluorescence-only) image. This is the preferred approach if a blank image is available. At least two images (one of which is a blank image) need to be selected before using this function. A dialog will appear and the users need to specify the blank image and the parameter values. We recommend starting with the default parameter values.

Help menu

The following options are available under the Help menu.

User's Guide

The software user manual is available under this option, and you can refer to detailed steps.

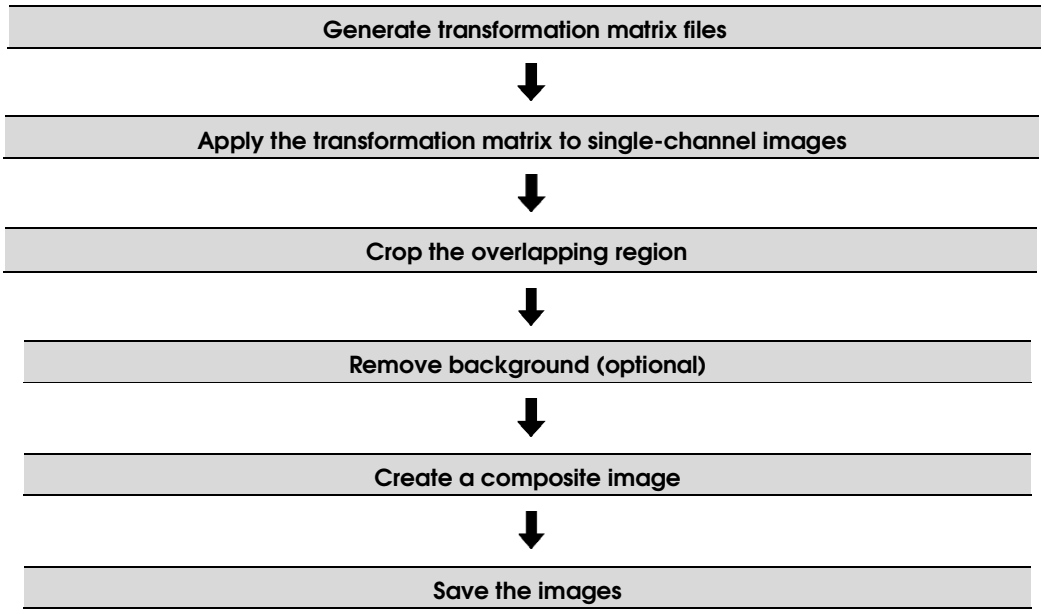
Sample Images

Use this function to review sample images provide by ACD. This feature enables you to familiarize yourself with the workflow of the RNAscope HiPlex Image Registration. You can use the R1_DAPI, R2_DAPI, and R3_DAPI samples images to generate the transformation matrix then later apply the matrix to single-channel images from Round 2 and Round 3.

4 Chapter 4. RNAscope HiPlex Image Registration

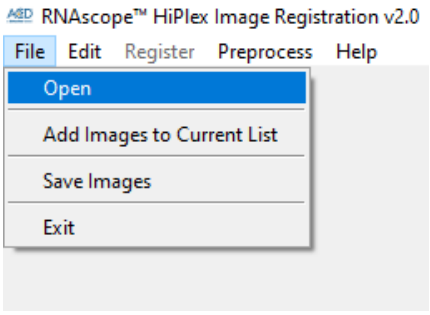
Follow the procedure in this chapter to register and merge your RNAscope HiPlex Assay images.

Workflow

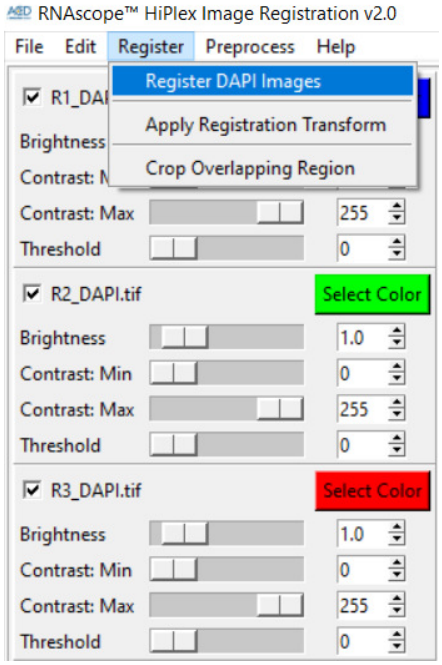


Generate registration transformation matrix files

1. Open the RNAscope HiPlex Registration software
2. From the File menu, click on Open. A pop-up window appears.

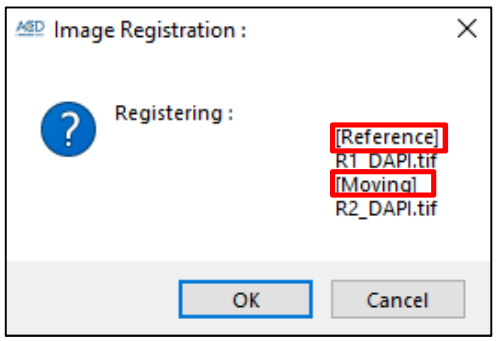


3. Open only the DAPI images from each round that need to be registered.
4. Select all the DAPI images you want to register by checking the box next to each image or clicking on Edit > Select All.
5. To register your selected images, click on Register > Register DAPI Images.

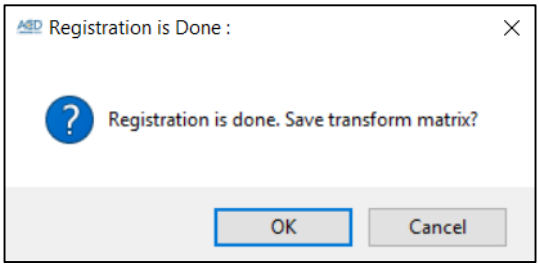


Create a transformation matrix for aligning DAPI from Round 2 onto DAPI from Round 1. By default, the first DAPI image in the list serves as the reference image. All other images will be registered to this image. The DAPI registration generates a transformation matrix in the form of a *tfm file.

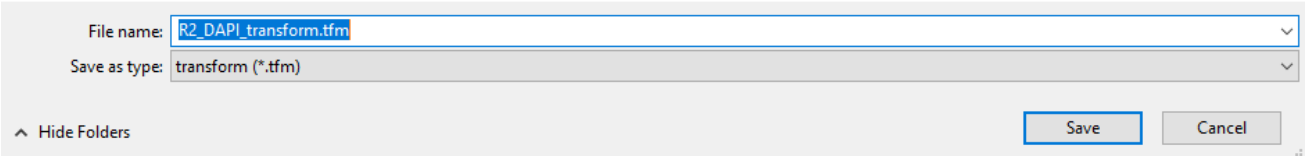
6. During registration, a pop-up window displays the file names of the reference image (Reference) and the moving image (Moving) being registered.



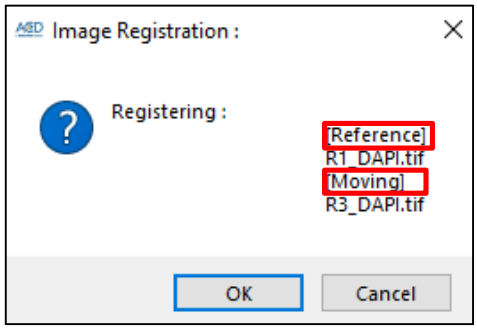
7. Click on OK to confirm registration and save the transformation file. Later, you will use the transformation matrix file to align the corresponding other single-channel images from the (Moving) Round onto the (Reference) Round.



8. Name the transformation matrix file. Make sure to indicate which image was merged. The software provides you with a suggested file name based on the file name of the original image.



9. If you are aligning more than two rounds, you will be prompted to save a transformation matrix that aligns DAPI from Round 3 to the DAPI from Round 1.

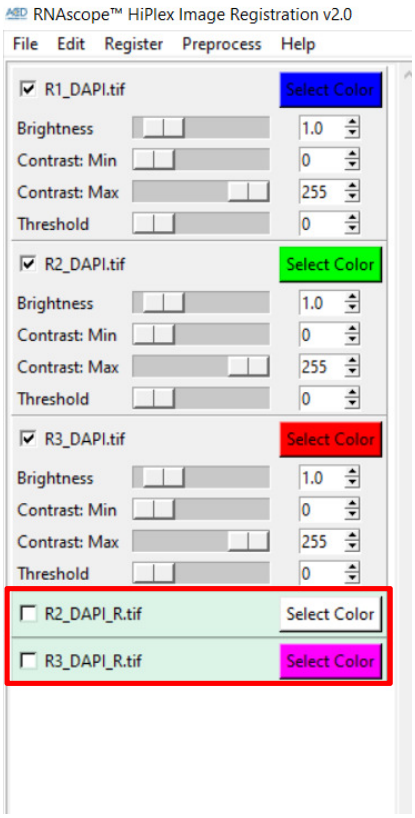


10. Click on OK to confirm registration and save the transformation file.
11. Name the second transformation matrix file. Make sure to indicate which image was merged. The software provides you with a suggested file name

based on the file name of the original image. Repeat steps 9-11 for additional rounds of DAPI.

Note: If you have an additional round of blank image acquisition (in this case, you may have 5 additional images in the "blank" round: blank DAPI, blank 488, blank 550, blank 650, and blank 750), treat this additional round the same way you treat any other round to generate an additional transformation matrix for blank image registration (i.e. include the blank DAPI image together with all other DAPI images in the above steps and generate an additional blank_DAPI_transform.tfm file).

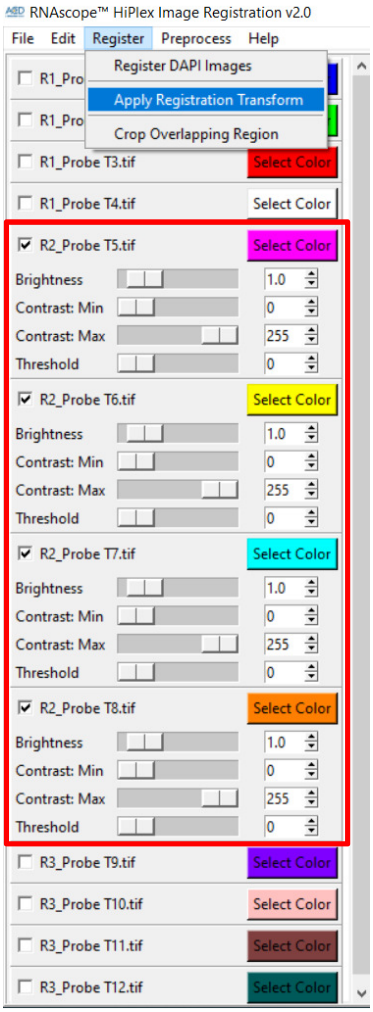
12. The newly registered images appear in the current list of images with **_R** added to the file name and a different background color. Make sure the registration was successful by overlaying the original DAPI images with the DAPI_R files. If the alignment looks good, use the DAPI_R.tif instead of the original DAPI channel images. If you overlay the original DAPI files and the DAPI_R files, you will have redundant DAPI.



13. Proceed to the next section to use the transformation matrix (*tfm) files created by registering the DAPI images to overlay the other single channels from each imaging round to the reference.

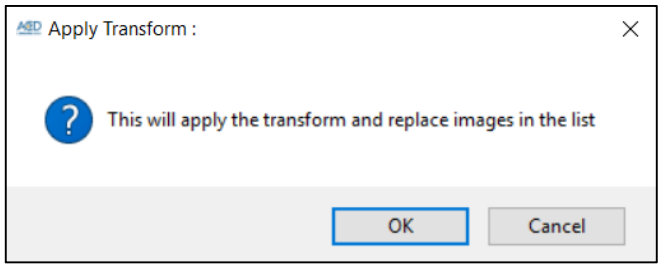
Apply the transformation matrix to single-channel images

1. From the File menu, click on Open to browse and open the source folder containing all of your single-channel images. This option overwrites the current list and opens fresh images. Alternatively, you can also use Add Images to Current List to open only a few images, which are added to the end of the list.
2. Since Round 1 was used as a reference for all the other rounds, do not apply the transformation matrix to any images from Round 1.
3. To apply the transformation matrix to your Round 2 images, check the boxes next to the Round 2 single-channel images, and click Register > Apply Registration Transform.

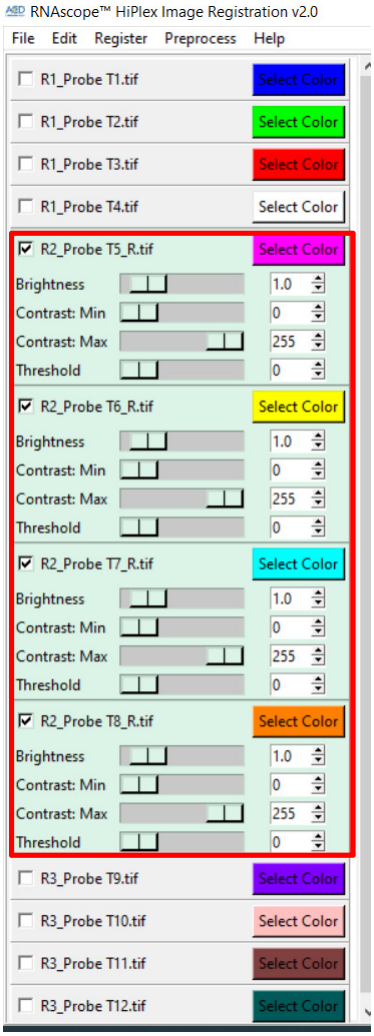


4. A pop-up window appears prompting you to select the appropriate transformation matrix file. Select the *.tfm file that corresponds with Round 2 (where Round 2 DAPI was moving with respect to the reference).

5. Click on Open to apply transformation matrix to all the selected images. When the prompt appears, click on OK to confirm the transformation process.



6. The newly registered images appear in the current list of images with **_R** added to the file name and a different background color.



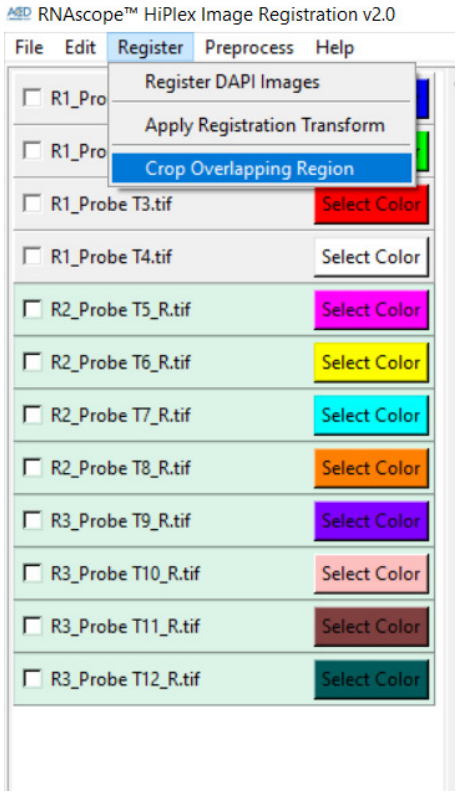
7. To apply the transformation matrix to your Round 3 images, first uncheck the Round 2 images. Check the boxes next to the Round 3 single-channel images, and click on Register > Apply Registration Transform.

8. A pop-up window appears prompting you to select the appropriate transformation matrix file. Select the *.tfm file that corresponds with Round3 (where Round 3 DAPI was moving with respect to the Reference).
9. Click on Open to apply the transformation matrix to all the selected images. When the prompt appears, click on OK to confirm the transformation process.
10. The software displays the list of registered images, with **_R** added to the file name and a different background color.
11. Repeat the same steps if you have more then three rounds of images.
12. If you have an additional round of blank image acquisition (in this case, you may have 5 additional images in the "blank" round: blank DAPI, blank 488, blank 550, blank 650, and blank 750), treat this additional round in the same way as you treat any other regular rounds to register all your blank images (i.e. use the blank_DAPI_transform.tfm file generated previously to register your blank 488, blank 550, blank 650, and blank 750 images).

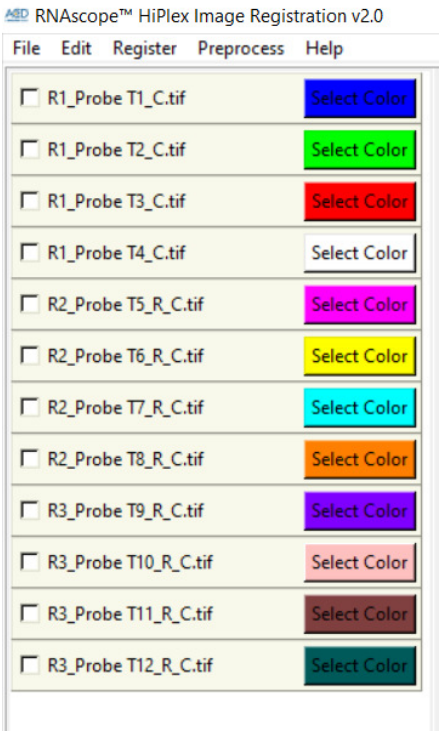
Crop the overlapping region

After applying the transformation and while the images are still open, you can automatically crop the maximum, overlapping region. This change is applied to all of the images in the list, including Round 1 images.

1. Click on Register > Crop Overlapping Region.

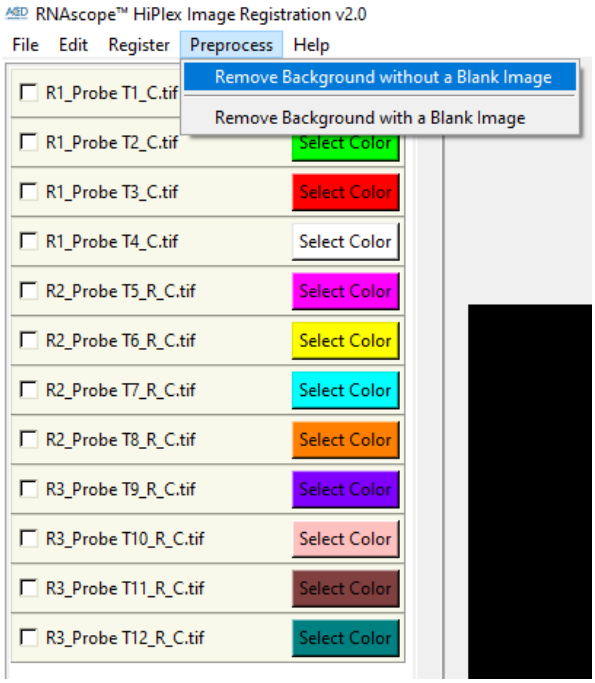


2. All images are updated with **_C** added to the file name and a different background color.

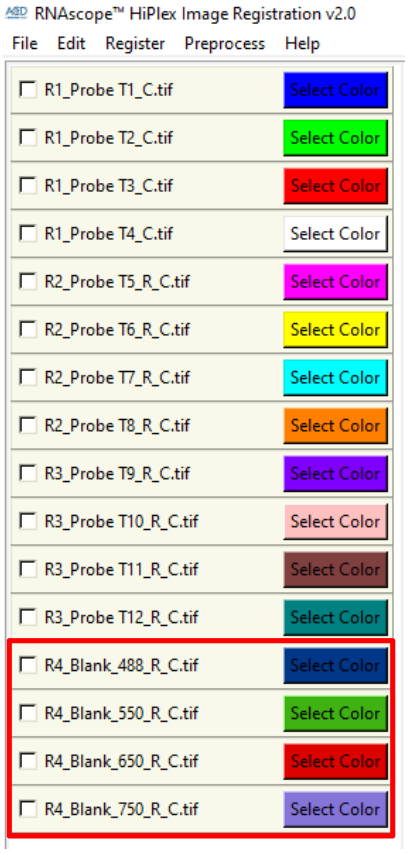


Remove background (optional)

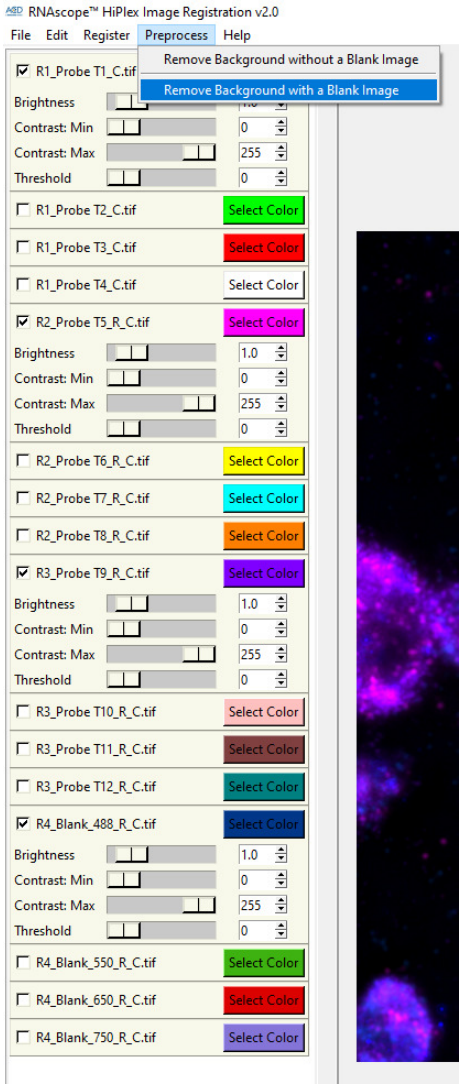
If you are handling images from FFPE samples or sample types with high autofluorescence, you may want to reduce the background from your images. This can be achieved by first selecting images and then clicking on Preprocess > Remove Background without a Blank Image or Preprocess > Remove Background with a Blank Image. If you have a blank image (autofluorescence-only image) available, we recommend using Remove Background with a Blank Image. The blank images can be acquired in a separate round of imaging after cleaving all the probes or before the probing process. In either case, there should be one blank image for each channel.



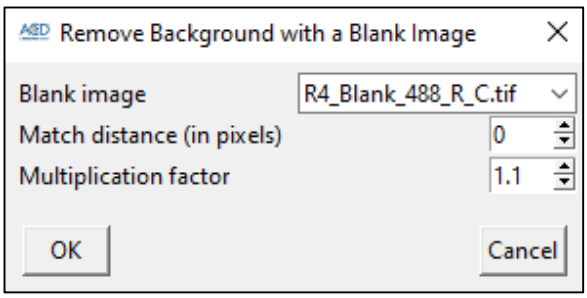
1. Remove Background without a Blank Image: This function provides single-image-based autofluorescence background reduction/removal. Only one single image, from which background removal is desired, needs to be selected before using this function. You can also select multiple images and background removal will be applied to all of them. After applying this function, new images with the background removed are created in the image list with **_BgR** added to the file name and a different background color.
2. Remove Background with a Blank Image:
    a. This function handles background subtraction using a blank (autofluorescence-only) image. This function requires an additional round of blank image acquisition, to which all previous steps (image registration and cropping) should also be applied in the same way as to a regular round. Assuming you have three regular rounds with an additional round of blank image acquisition, your image list may look like the following before the background removal step:



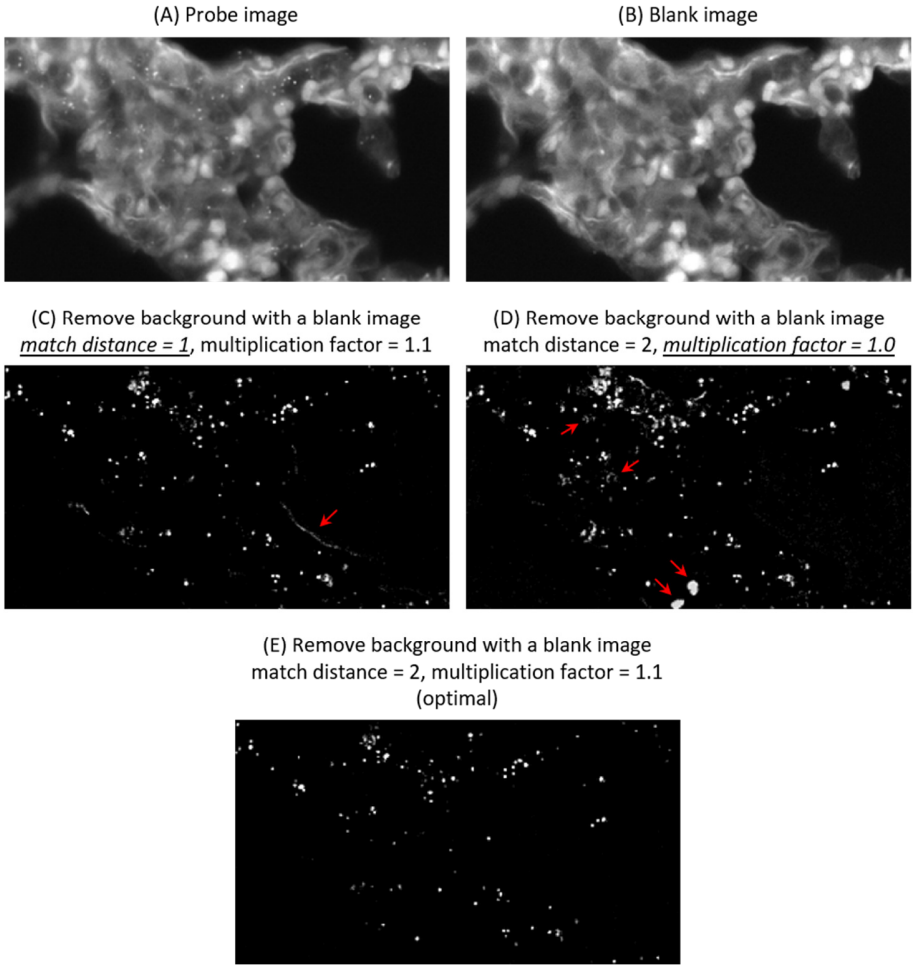
b. At least two images (one of which is a blank image) need to be selected before using the Remove Background with a Blank Image function. When selecting more than two images, background removal will be applied to all except the blank image. When selecting images, keep in mind that all images acquired in the same channel across different rounds should be selected. In the following example, images acquired under the AF488 channel are selected.



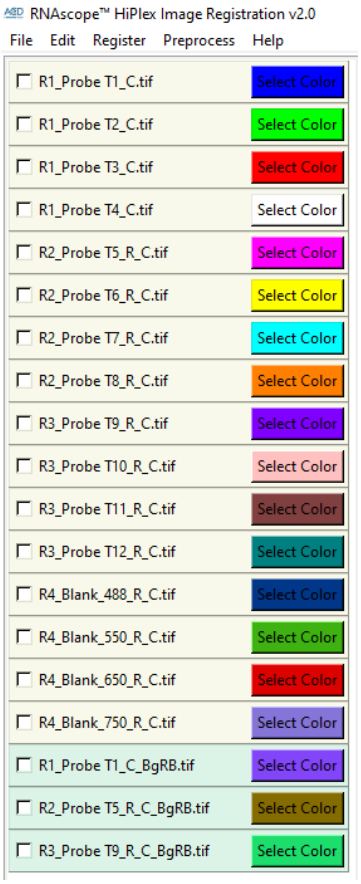
c. After clicking Remove Background with a Blank Image, a dialog will appear and the users need to specify the blank image (by default the last image will be assigned as the blank image) and the parameter values. We recommend starting with the default parameter values. Consider the following when you need to adjust parameter values:



- Match distance (in pixels): This is used to indicate locally how much distance the blank image has to shift to match the probe image. Note that this only refers to local mismatches; all global mismatches have already been corrected by registration. If the probe and blank images have a perfect match, you can set this value to zero. Increase the value of this parameter if you still have remaining background at the edge of the autofluorescence pattern following the use of Remove Background with a Blank Image. Try adjusting this parameter first; if you have achieved satisfactory results, we recommend that you keep the other parameter (Multiplication factor) unchanged. See the following example image and compare panel C vs. panel E. Check the remaining background indicated by the red arrow in panel C as a guide when adjusting this parameter.
- Multiplication factor: This factor will be multiplied to the blank image before subtraction, to help compensate for any additional local intensity variations in the blank image. Increase the value of this parameter if you still have remaining background inside the autofluorescence pattern following the use of Remove Background with a Blank Image. See the following example image and compare panel D vs. panel E. Check the remaining background indicated by the red arrows in panel D as a guide when adjusting this parameter.



d. After applying Remove Background with a Blank Image, images with the background removed are created in the image list with **_BgRB** added to the file name and a different background color.

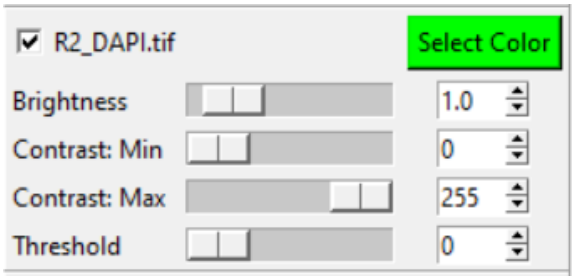


Create a composite image

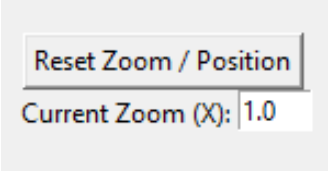
1. Before you save the images, create composite images by selecting multiple channels.
2. When a channel is selected, its display adjustment controls appear. A pseudo color is assigned to each image automatically. You can change the assigned color.
3. All of the four display adjustment parameters (Brightness, Contrast: Min, Contrast: Max, and Threshold) can adjust image display in real time. There are three ways to change the value of each: the slider, by directly entering the values, and fine-tuning using the two arrow-head buttons. See the following for further details:
    - Brightness: Adjusts the overall intensity for each channel.
    - Contrast: Min and Contrast: Max: Enhances the contrast of the pixels with values in between them. Pixel values below or equal to Contrast: Min are changed to the lowest pixel value for display while pixel values above or equal to Contrast: Max are changed to the highest pixel value for display. Pixel values between Contrast: Min and Contrast: Max

are then remapped to the entire display range and receive the best contrast.

- Threshold: For pixels with values below Threshold, zero values are assigned to them for display. All pixels with values above or equal to Threshold remain unchanged.

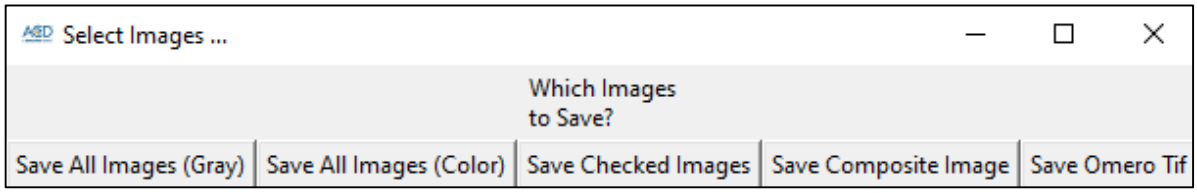


4. To change the image zoom, use the mouse wheel when your mouse pointer is hovering over the image display area. Use the following to help control the zoom:
    - Reset Zoom/Position button: When this button is pressed, the image display returns to the default position in the display window with zoom = 1X.
    - Current Zoom (X): This box indicates the current zoom magnification. The value in the box changes when the zoom is changed using the mouse wheel. You can also change the zoom by typing in a valid entry. If an invalid value is entered, the closest value in the valid range is used.



Save the images

1. To save the images, click on File > Save Images.
2. From the pop-up options menu, choose whether to save each image as gray scale or color. Please note that it is preferable to save the images as gray scale such that the original pixel intensity values are saved for further quantification analysis. You can choose to save all the images or select the images you want to save.



Troubleshooting

For troubleshooting information, please contact technical support at support.acd@bio-techne.com.

Documentation and support

Obtaining support

For the latest services and support information, go to: www.acdbio.com/technical-support/support-overview.

At the website, you can:

- Access telephone and fax numbers to contact Technical Support and Sales facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Search for user documents, MSDSs, application notes, citations, training videos, and other product support documents.
- Find out information about customer training events.

Contact information

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Limited product warranty

Advanced Cell Diagnostics, Inc. and/or its affiliate(s) warrant their products as set forth in the ACD General Terms and Conditions of Sale found on the ACD website at www.acdbio.com/store/terms. If you have any questions, please contact Advanced Cell Diagnostics at www.acdbio.com/about/contact.

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