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OVERVIEW OF APPLICATION

The purpose of this user interface is to streamline user-assisted registration of multi-plex biological images for the purpose of co-registering coordinate data. To use this interface, the user should first define a project. Each project consists of a single fixed image and one or more moving images. All moving images will be aligned to spatially overlay on the fixed image. The registration transforms calculated to align the moving image to the fixed image may then be used to register coordinate data. Often, the fixed image should be a whole slide H&E, but this can vary. All images used here should be downsampled from their original resolution to a size enabling rapid loading and manipulation (<150MB). The user must know the scale between the downsampled image and the full resolution file.

To better enable down-sampling of diverse image file-types we provide a QuPath-based script.

[IAN TO ADD A BRIEF DESCRIPTION OF THE QUPATH DOWNSAMPLING SCRIPT HERE]

Before you begin, make a list of all images in your cohort. For each image, you need:

- The original, full resolution copy of the image
- A downsampled image that can be rapidly loaded into computer memory for the registration
- The scale factor between the downsampled image and the full resolution file
- Coordinates corresponding to information you quantified in the full resolution image.

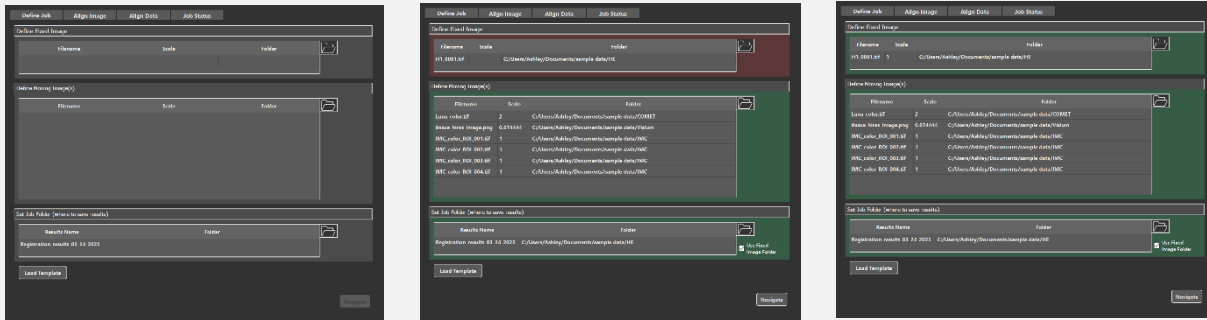
TAB 1. DEFINE PROJECT

The purpose of this tab is to enable users to set up a registration project by defining their fixed and moving images and designating the folder to save the registration outputs. To complete this tab, follow the steps below.

STEP 1. DEFINE THE INPUTS TO YOUR PROJECT

There are two methods to input data. **Option 1.** define each required input manually or **Option 2.** load the inputs from a previous project. When all inputs are defined correctly, the panel will turn **green**. If any input is incomplete or defined incorrectly, the panel will turn **red**. The user may advance to the next tab when all panels are **green**.

Panels will highlight in red if incorrect and green if completed



OPTION 1. DEFINE THE INPUTS MANUALLY

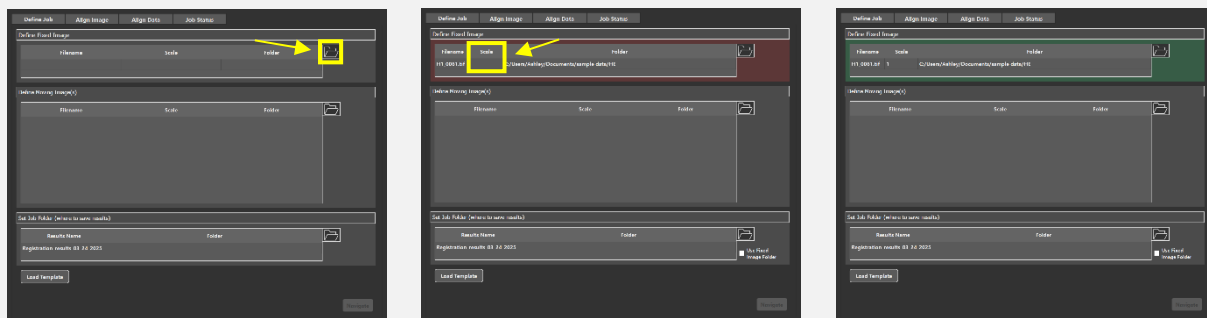
The user may define the project inputs manually by completing the three panels shown.

STEP 1A. COMPLETE THE "DEFINE FIXED IMAGE" PANEL

Select the folder icon button inside the fixed image panel and navigate to the folder containing a downsampled copy of the image that will serve as the reference coordinate system for this project. Select the image, which will populate the "Filename" and "Folder" cells of the table. The panel will turn **red** as the "Scale" cell needs to be defined. Double click the "Scale" cell and input the factor to up-sample the image back to full resolution. For example, for a full resolution image of 21,000 x 21,000 downsampled to 3,000 x 3,000 pixels, the scale factor is $21,000 / 3,000 = 7$.

To replace the current fixed image with another image, select the Folder icon button and confirm replacement of the current file. Repeat the process to locate the desired file and enter the scale factor.

Import the downsampled fixed image and define the downsample scale factor

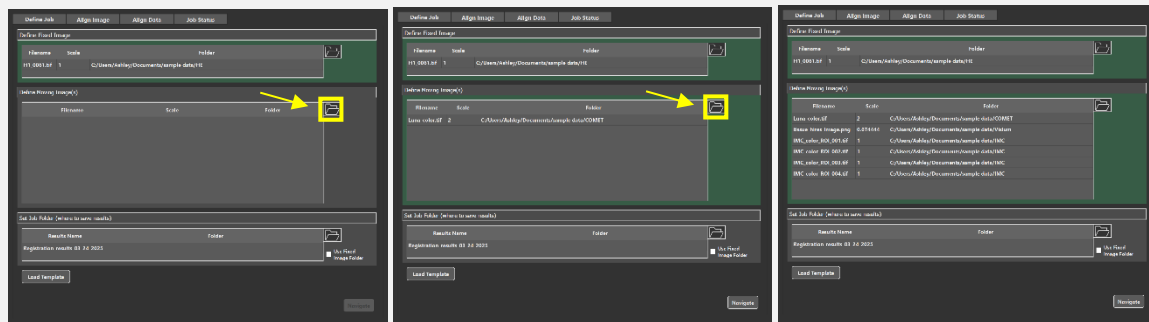


STEP 1B. COMPLETE THE "DEFINE MOVING IMAGE(S)" PANEL

Select the folder icon button inside the moving image panel and navigate to a folder containing a downsampled copy of an image that will be registered to the fixed image. Select the image, which will populate the "Filename" and "Folder" cells of the table. The box will turn **red** as the "Scale" cell needs to be defined. Define the scale between the downsampled moving image and the full resolution file such that the box turns **green**. Continue adding moving images by repeating this process until all desired images are defined.

To delete a moving image from the table, double click the filename of the image you wish to delete, then press the "Delete" button when it appears.

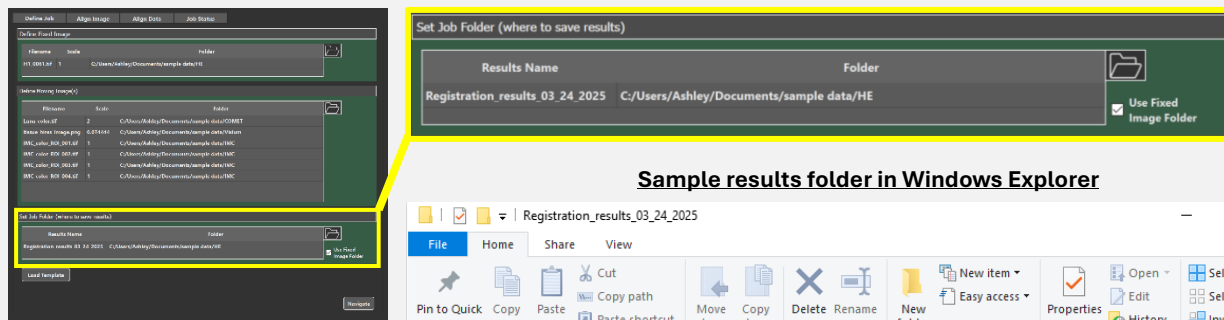
Import the downsampled moving images and define the downsample scale factors



STEP 1C. COMPLETE THE "WHERE TO SAVE RESULTS" PANEL

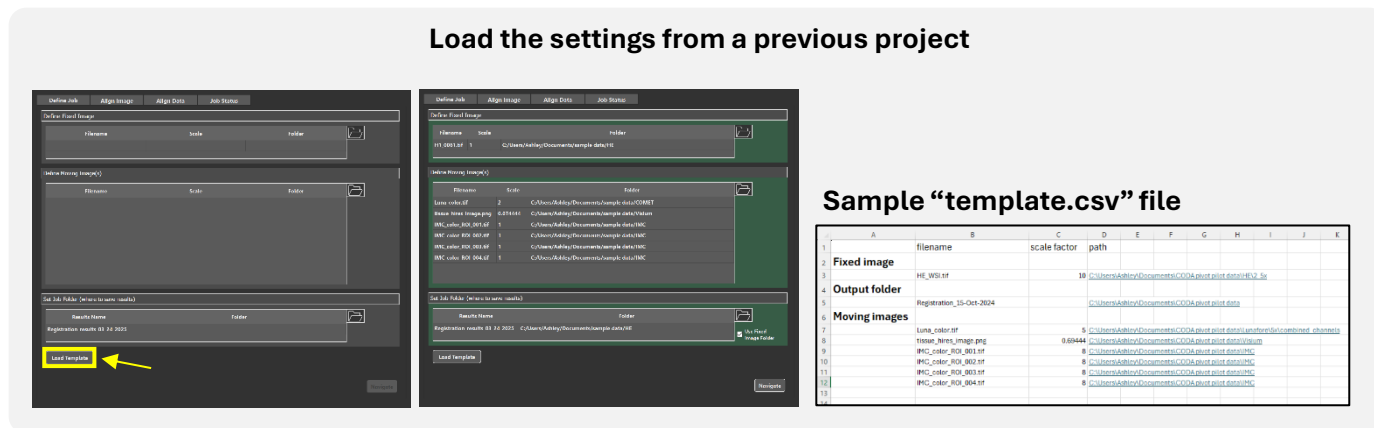
Finally, define the folder where the registration results should be saved. Either select the "Choose folder" button and browse for a folder or check the "Use fixed image folder" checkbox to save the results in the same folder as the fixed image. By default, the registration results name is set to "Registration_[TODAY'S DATE]", but this may be changed by double clicking the "Results name" cell in the table and defining a custom job name. The outputs of this project will be saved inside of a folder with the results name in the defined results folder.

Define the project name and choose where to save the registration project results



OPTION 2. LOAD INPUTS FROM A PREVIOUS PROJECT

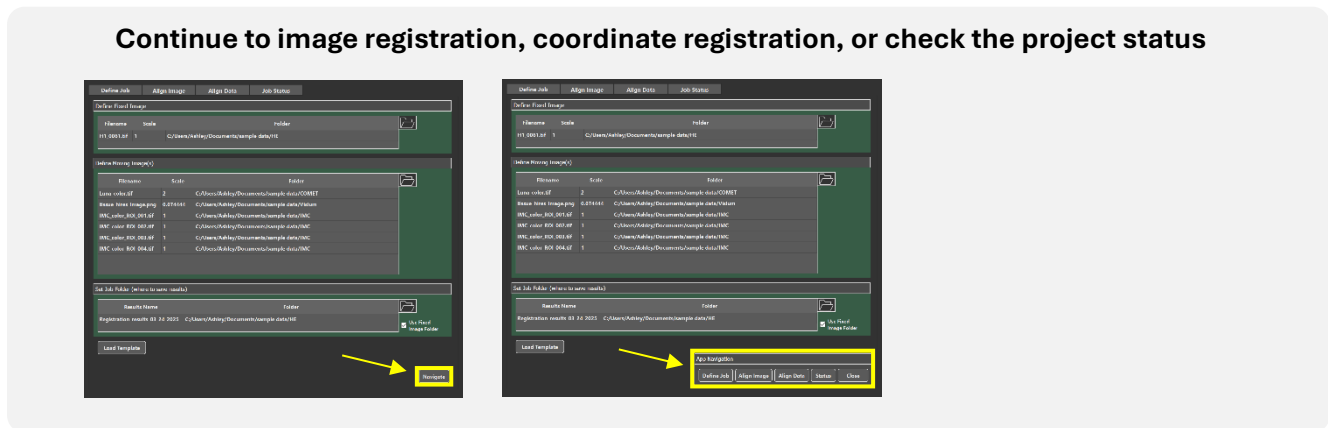
Where a user has previously started a project and would like to continue or modify it, the user may reload previous inputs. Select the "[Load template](#)" button on the bottom left corner of the tab. Navigate to the folder containing the registration results of the desired project and select the "[template.csv](#)" file. This will auto-populate the three panels, which will turn green if all inputs are defined correctly. Edit these inputs as desired.



STEP 2. NAVIGATE ACROSS VARIOUS TABS TO PERFORM IMAGE OR COORDINATE REGISTRATION

Once all inputs are defined correctly, the user may move across the tabs of the interface using the “[Navigate](#)” button. When the user navigates away from this tab, the inputs will be saved in the job folder as a “template.csv” file. If the user exits the interface and rejoins, this project may be continued by importing the template.

1. To view the project settings, select the “[Define Job](#)” button to proceed to **Tab 1: Define Project**.
2. To compute image registration between the fixed and moving images, select the “[Align Images](#)” button to proceed to **Tab 2: Align Images**.
3. To apply previously calculated registration transforms to coordinate data, select the “[Apply Data](#)” button to proceed to **Tab 3: Apply registration data**. Note: In order to apply transforms to coordinate data, images must first be registered via the “[Align Images](#)” button. Select “[Align Data](#)” only if you have previously registered your images in this project and now seek to apply those existing results to data.
4. To view the current progress, select the “[Status](#)” button to proceed to **Tab 4: Project Status**. This tab contains a table summarizing the relevant image and coordinate registration information for the current project.
5. The below sections define the layout and functionality of tabs 2 – 4.



TAB 2: ALIGN IMAGES

The purpose of this tab is to enable users to view the fixed image and moving images side-by-side to guide rapid and high-accuracy fiducial point placement. Following fiducial point placement, this tab enables point-cloud-based affine registration of the fixed and moving images, followed by optional calculation of nonlinear registration to account for user-error in fiducial placement and local distortion (pulling or folding) within the structures of the images. To complete this tab, follow the steps below.

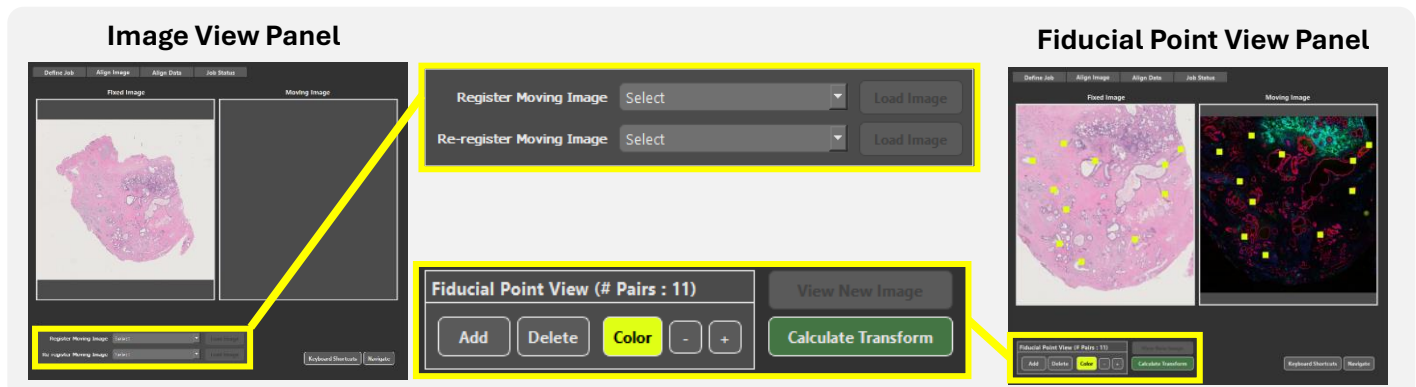
STEP 1. SELECT A MOVING IMAGE TO REGISTER (OR RE-REGISTER)

Upon start-up, this tab will display the fixed image on the left, and a blank frame on the right. The “[Navigation](#)” button on the bottom right corner of the tab may be used at any time to move to another tab of the interface.

To load a moving image, select a file from either the “[Register moving image](#)” or “[Re-register completed moving image](#)” dropdown menus on the bottom left corner of the tab. To display a selected image, select the “[Load image](#)” button. The image will load and display on the right image panel. If a previously registered image was selected, existing fiducial pairs and view settings will be present.



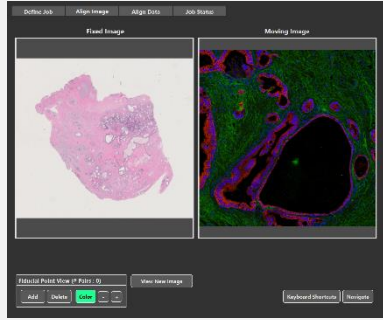
When a moving image is loaded, the “[Fiducial point controls](#)” panel will replace the “[Moving image selection panel](#)” in the bottom left corner. To the right of the “[Fiducial point controls](#)” panel two buttons will appear: “[View new image](#)” and “[Calculate registration.](#)” Select the “[View new image](#)” button anytime to close the current moving image and choose another. The “[Calculate registration](#)” button will be disabled until at least 6 fiducial pairs are clicked. If enabled, this button will prompt calculation of point-cloud registration of the fixed and moving image.



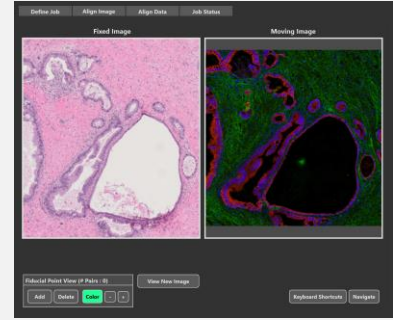
STEP 2. MANIPULATE FIXED AND MOVING IMAGE VIEWS TO DISPLAY SIMILAR REGIONS

After the moving image is displayed, the user may apply various transformations to the images with the goal of displaying the same region of interest in each panel and adjusting the brightness and contrast to facilitate efficient fiducial pair placement. Select the “[Keyboard Shortcuts](#)” button in the bottom right corner to view the available transformations (and see them copied below in Table 1). Use these commands to adjust the fixed and moving image if necessary to visualize the same region of interest.

View the Keyboard Shortcuts pop-up window and adjust the image views



ROTATE (r), ZOOM (scroll), and PAN (click & drag) the fixed image to identify the same ROI as the moving image



Command	Action			
Zoom	Hover mouse over desired image and scroll			
Pan	Click and drag on desired image			
Save a screenshot	S			
	Left Image		Right Image	
Rotate +90°	R		Shift + R	
Flip on vertical axis	F		Shift + F	
Auto-adjust contrast	A		Shift + A	
Return to default view	D		Shift + D	
	Small effect	Large effect	Small effect	Large effect
Increase brightness	B + >	B + L	Shift + B + >	Shift + B + L
Decrease brightness	B + <	B + K	Shift + B + <	Shift + B + K
Increase contrast	C + >	C + L	Shift + C + >	Shift + C + L
Decrease contrast	C + <	C + K	Shift + C + <	Shift + C + K

Table 1: Image View Command Shortcuts

STEP 3. SELECT FIDUCIAL POINT PAIRS

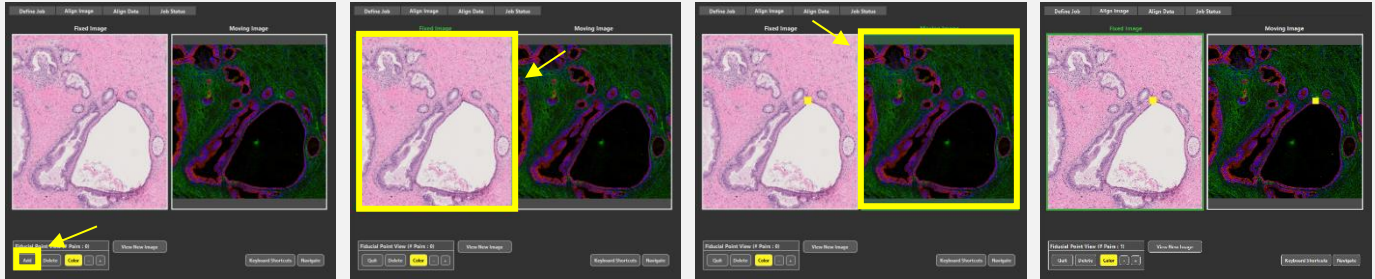
Once the fixed and moving images display similar regions of interest, fiducial point selection may begin.

Note: We suggest a minimum of 7 fiducial points for high quality registration. As the accuracy of the point-cloud-registration depends on the accurate placement of fiducial pairs, we strongly suggest users utilize the zoom and pan features to select fiducials at a high magnification.

STEP 3A. ADD FIDUCIAL POINT PAIRS

To add a point pair, select the “Add” button in the “Fiducial point controls” panel. The fixed image will be highlighted in **green**, and the mouse icon will be replaced by crosshairs. The user should click a fiducial landmark in the fixed image. Next, the moving image will be highlighted in **green**. The user should click **the same structure** in the moving image as was selected in the fixed image. Following selection of the paired point, the app will switch back to the fixed image to allow the user to define another point pair. Continue selecting point pairs until the desired number (minimum of 6) is obtained. Image View commands are active during fiducial selection, enabling users to zoom, pan, rotate, and flip images to better support accurate point placement. When the desired number of points have been selected, click “esc” or press the “Quit” button to exit fiducial point selection mode.

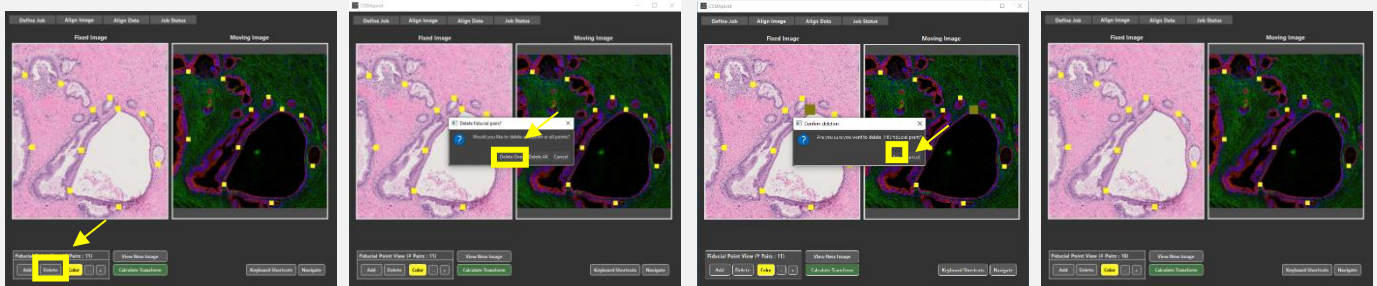
Add fiducial point pairs to the fixed and moving images



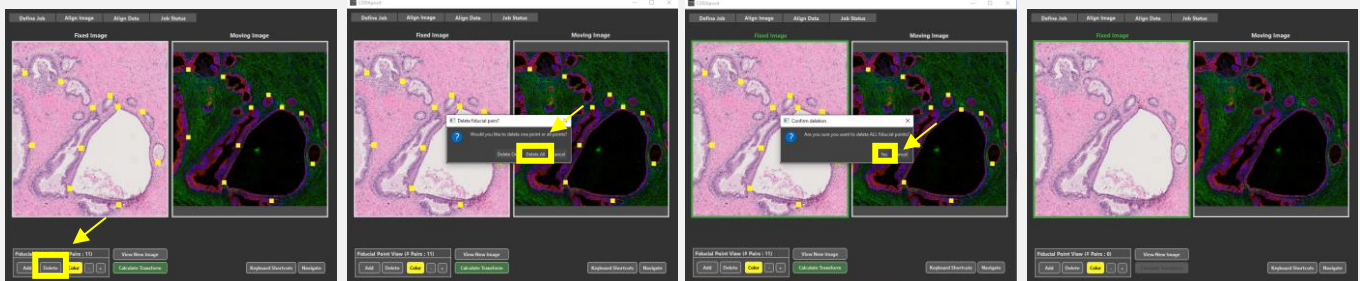
STEP 3B. DELETE FIDUCIAL POINT PAIRS

To remove a fiducial point pair, select the “Delete” button in the “Fiducial point controls” panel. Select “Delete one point” or “Delete all points” in the pop-up window as desired. If “Delete one point” was selected, the mouse icon will be replaced by crosshairs. Click near the point you wish to delete in either the fixed or the moving image. The nearest point to the coordinates clicked will be highlighted, and the user will be asked to confirm or cancel the deletion. If confirmed, the selected point, and its corresponding paired point in the other image, will be deleted.

Delete a single fiducial point from the fixed and moving images



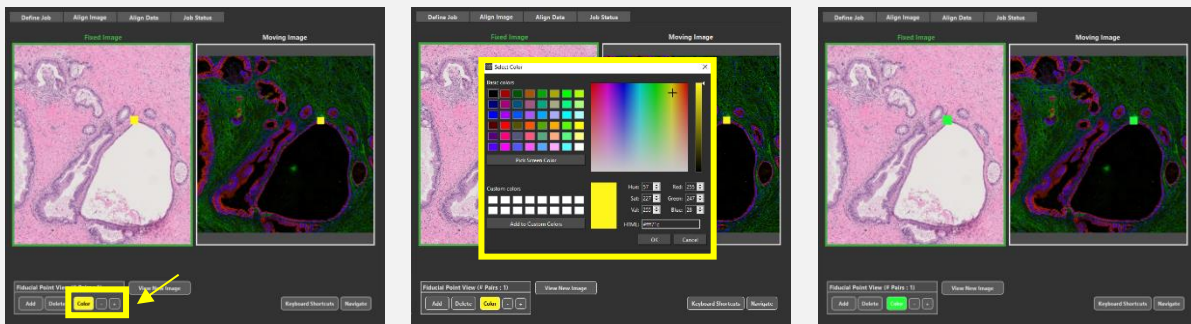
Delete all fiducial points from the fixed and moving images



STEP 3C. FIDUCIAL POINT VISIBILITY

To improve marker visibility, the user may change the “Color” or “Size” of the points via the corresponding buttons within the “Fiducials point controls” panel.

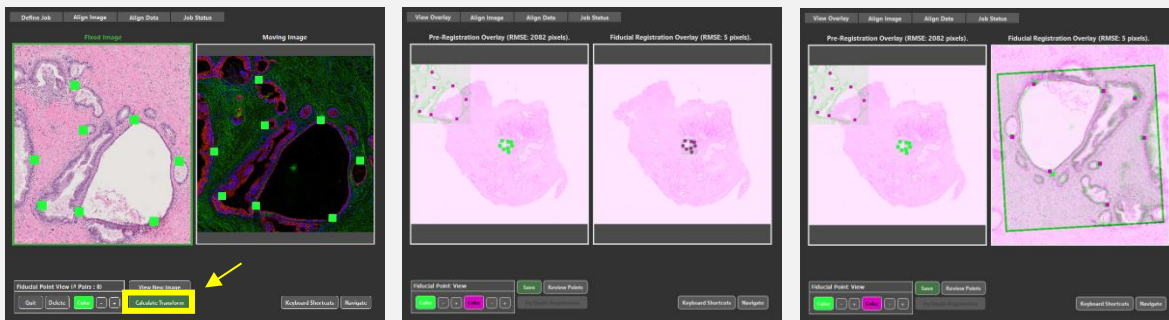
Change the color and size of the fiducial points



STEP 4. CALCULATE POINT-CLOUD-BASED IMAGE REGISTRATION

Once the user has annotated a minimum of six fiducial point pairs, the “Calculate registration” button will be enabled. Select this button to calculate the point-cloud-based affine registration, and apply the affine transform to the moving image. Here, the selected fiducial point pairs and view settings will be saved to a “.pkl” file. The user will be directed to a tab displaying to compare the pre- and post-registration image overlays.

Save fiducial point coordinates and calculate registration. Zoom to view results



STEP 4A. DETERMINE WHETHER THE POINT-CLOUD-BASED REGISTRATION IS ACCEPTABLE

The layout of the image overlay sub-tab should look similar to tab two, with images displayed on the left and right side of the screen. Displayed are overlays of the fixed image (in **green**) and the moving image (in **pink**), with their corresponding fiducial points. On the left side of the screen is the overlay of the **unregistered** images, and on the right is the overlay of the **registered** images. In the text above the left and the right image panel the root mean squared error (RMSE) of the unregistered and registered fiducial point pairs is printed. An RMSE of 0 represents perfect alignment, while a large RMSE represents poorly aligned images. In the below example, the original RMSE of 32,647 pixels is reduced to 4 pixels through the point cloud registration.

As during fiducial pair placement, the user may edit the view of the image overlays using the keyboard shortcuts, and may change the size or color of the fiducial point pairs to improve the interpretability of the results. The user should review the image and fiducial point pair overlays to determine whether the alignment is of sufficient quality.

STEP 4B. SAVE THE POINT-CLOUD-BASED REGISTRATION RESULTS OR RETURN TO EDIT FIDUCIAL PAIRS

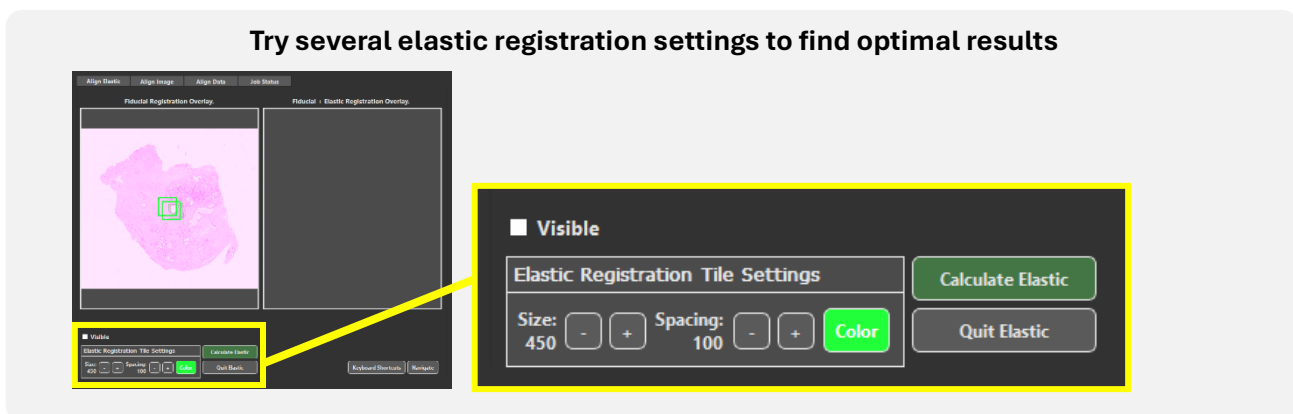
After assessing the quality of the registration by reviewing the pre- and post-registration RMSE and visualizing the image overlay, the user can either save the calculated registration transformation matrix by selecting the “[Save](#)” button, may return to adjust their fiducial point pair selection by selecting the “[Edit Results](#)” button, or may move to another tab altogether by selecting the “[Navigate](#)” button.

The “[Save](#)” button is highlighted in **green** if the user has not yet saved these results, and will turn **grey** after the results have been saved. If the results are saved, the “[Calculate Elastic Registration](#)” button will be enabled and will turn **green**. If desired, the user may select this button to attempt elastic registration to finetune the results of the affine registration. Alternatively, the user may select the “[Navigate](#)” button to move to another tab.



STEP 5. CALCULATE ELASTIC IMAGE REGISTRATION (OPTIONAL)

If the “[Calculate Elastic Registration](#)” button was selected, the user will be taken to another tab, this one enabling the user to adjust parameters and calculate the elastic image registration. Again, there will be two image panels. The left panel will be populated with an overlay of the point-cloud-based registration results, and the right panel will be empty. Within the left panel, there will be two square outlines present, representing the size and spacing of tiles that will be used for the elastic registration calculation. The user may adjust the size and spacing of these tiles, or change their color to improve visibility, using the buttons inside the “[Elastic registration controls](#)” panel. For brightfield to brightfield registration (H&E to H&E or H&E to IHC) we suggest a tile size of 250 – 350. For brightfield to fluorescent, we suggest a larger tile size of 400-600.



To calculate registration using the default or selected parameters, select the “[Calculate transform](#)” button. This will initiate tile-wise calculation of image registration, which will be concatenated and interpolated to generate a

nonlinear transformation matrix. Select the ‘Visible’ checkbox to view the tile-wise progress of the algorithm while it calculates. Following calculation of the nonlinear transform, the moving image will be transformed, and the inverse of the transform will be determined for registration of the fiducial markers and for downstream application to coordinate data. Once the fiducial points and moving image are transformed, they will be overlaid with the fixed image in the right panel, and the RMSE of the elastic registration will be displayed above the image panel.

STEP 5A. DETERMINE WHETHER THE ELASTIC REGISTRATION IS ACCEPTABLE

As in the overlay for the point-cloud-based registration, the user should review the image and fiducial point pair overlays to determine whether the elastic alignment is of sufficient quality. Here, the user should note whether the RMSE improved from the point-cloud-based registration to the elastic registration and should review the image overlays to determine whether the moving image was improperly warped. If warping is present, we suggest the user calibrate the elastic registration by increasing the tile size. If the results are not locally warping enough, we suggest the user calibrate the elastic registration by decreasing the tile size. As the tile spacing can have non-intuitive effects on the registration results, we suggest users test a few values to optimize to their dataset.

STEP 5B. SAVE THE ELASTIC REGISTRATION RESULTS OR RETURN

After assessing the quality of the registration by reviewing the pre- and post-registration RMSE and visualizing the image overlay, the user can either save the calculated nonlinear displacement field by selecting the “Save” button, may return to adjust the elastic registration settings by selecting “Review Tiles” button, may return to the sub-tab overlay of the point-cloud-based registration results by selecting the “Quit Elastic” button, or may move to another tab altogether by selecting the “Navigate” button. The “Save” button is highlighted in green if the user has not yet saved these results and will turn grey after the results have been saved.



The purpose of this tab is to enable users to import coordinate data and align it to fixed image space using the transforms calculated from the fiducial point annotation and elastic registration. To complete this tab, follow the steps below.

The first step of this tab is to identify the coordinates to register and to define which moving image they correspond to. Select the Folder icon button to identify the “.csv” or “.xlsx” file containing the coordinate data. Inside this file, each row should contain information for one coordinate. The “x” coordinate should be listed in one column and the “y” coordinate should be listed in a separate column (see a sample coordinate file below). By selecting a coordinate file the “Data filename” cell in the table will be populated.

	A	B	C	D	E	F	G	H
1	barcode	in_tissue	array_row	array_col	pxl_row_in_fulres	pxl_col_in_fulres		
2	GTCACTTCC	0	0	0	1300	969		
3	CACGCTCTC	0	0	2	1300	980		
4	ATAGCTGCC	0	0	4	1300	991		
5	GTCAGTATC	0	0	6	1300	1002		
6	ATGTACCAC	0	0	8	1300	1014		
7	ACGCTCAGT	0	0	10	1300	1025		
8	TCACCTAACG	0	0	12	1300	1036		
9	CGGTTAGGC	0	0	14	1300	1047		
10	GATATCACC	0	0	16	1300	1058		
11	TCGCCTTGT	0	0	18	1300	1069		
12	CATACCTAC	0	0	20	1300	1080		
13	TATCCGGA	0	0	22	1300	1091		
14	CTCCAAGTC	0	0	24	1300	1102		

Double click the “X column” and “Y column” cells to input the letter or number of the column corresponding to the coordinates. For example, in the sample template file below, the coordinate rows are defined in column “E”, so the user may enter “E”, “e”, or “5”. Similarly, the coordinate columns are defined in column “F”, so the user may enter “F”, “f”, or “6” to the “Y column” cell. Once the “Data filename”, “Moving image filename”, “Scale”, “X column”, and “Y column” are defined, the panel will turn **green** to indicate the coordinates and moving image file may now be read into memory. Select the “Load” button to load the coordinates and corresponding images.

STEP 2. CONFIRM THE COORDINATES OVERLAY ON THE UNREGISTERED MOVING IMAGE

When all inputs from **STEP 1** are defined correctly, the user may press “**Load**” to read the coordinates and corresponding images into memory. The unregistered image will be displayed in the panel on the left side of the tab and the coordinates will be overlaid as a scatter plot. The user should confirm that the coordinates correctly overlay on the image or select the “**Edit Table**” or “**Swap XY**” buttons to edit the coordinates appearance if they do not correctly overlay. The user may adjust the view of the image using the previously described keyboard shortcuts, and may adjust the point data color and size using the “**Fiducial Point View**” tab under the image panel. When the coordinates correctly overlay on the image, select check the box next to the “**Unregistered moving image**” button.

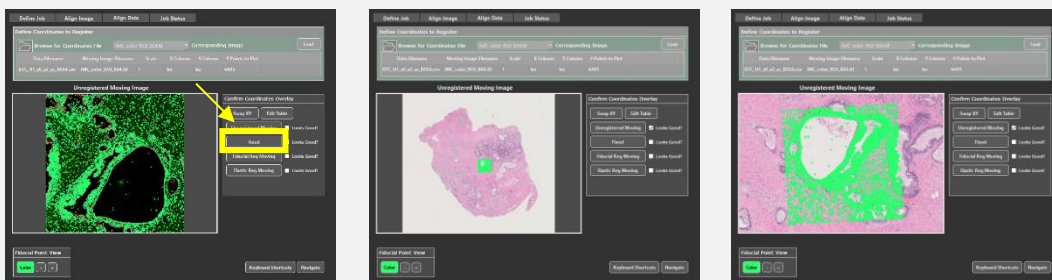
Confirm the correct overlay of the coordinates on the unregistered moving image



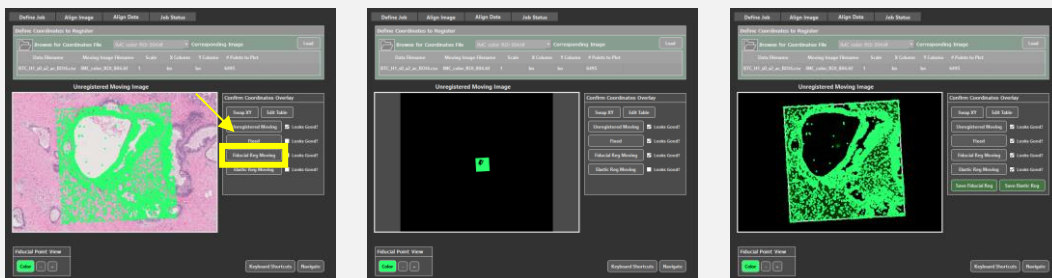
STEP 3. CONFIRM COORDINATES OVERLAY CORRECTLY IN REGISTERED SPACE

Next, select the “**Fixed Image**,” “**Registered moving image**”, and “**Elastic moving image**” (if elastic registration was calculated, to confirm that the registered coordinates overlay as expected. The user should check each overlay carefully to confirm that the registration is of sufficient quality for downstream analyses. If the quality is not sufficient, the user may select return to adjust the fiducial points and recalculate the registration by selecting the “**Navigate**” button on the bottom right corner of the tab. If the overlays are satisfactory for each button selected, check the corresponding checkbox to the right of the button to confirm correct overlay.

View the registered coordinate overlay on the fixed image

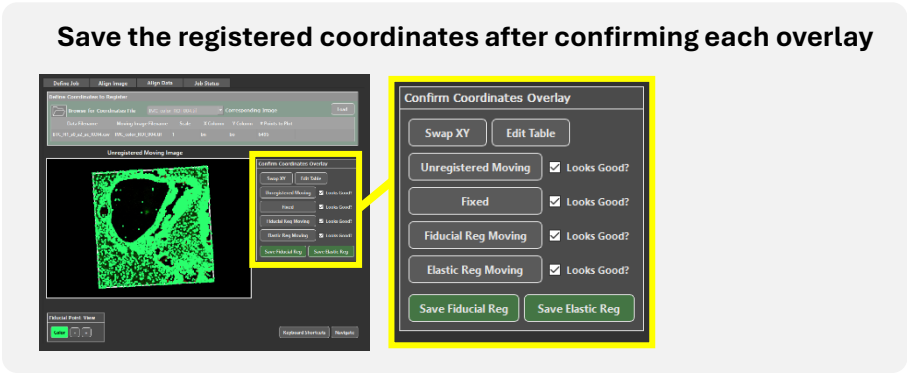


View the registered coordinate overlay on the point-cloud or elastic registered moving image



STEP 4. SAVE THE REGISTERED COORDINATES

Once all boxes are checked, two buttons will appear allowing the user to save the registered coordinates: “[Save Affine Reg](#)” and the “[Save Elastic Reg](#)” (if the elastic registration was calculated). Either or both of the affine and elastic registered coordinates may be saved. The coordinates will save inside the output folder defined in **Tab 1**. The filename will be the same as the input filename, appended with either “[Registered](#)” or “[Elastic registered](#)” depending on which button was selected. The format of the file will be the same as the input format, with the unregistered coordinate values replaced with registered coordinate values at the scale of the full resolution fixed image as defined in **Tab 1**. After saving the coordinates, the user may move to another tab in the interface or restart this tab to register more coordinate files by selecting the “[Navigate](#)” button.



TAB 5. VIEW PROJECT STATUS

The purpose of this tab is to enable users to view the status of their project. No actions are completed in this tab, but a table is present which will display the name of the fixed image, the name of each moving image, and for each moving image: the number of fiducial pairs that have been saved, the calculated RMSE for the point-cloud-based and elastic registration, and whether or not coordinate data has been registered with the resulting registration transforms. This tab may be used at anytime to view the progress and validation (RMSE) of the project.

View the project status

Define JobAlign ImageAlign DataJob Status

Job Status for each Moving Image

Image Name	# Fiducials	I/P Registration	Elastic Registration	Coordinates Registered
Fixed Image: H1_0001.tif	-	-	-	
Moving Image 1: Lung_color.tif	12 pairs	RMSE: 7 pixels	RMSE: 7 pixels	
Moving Image 2: Brain_hires_image.png	9 pairs	RMSE: 31 pixels	RMSE: 35 pixels	Saved!
Moving Image 3: BMC_color_RCI_001.tif	0 pairs			
Moving Image 4: BMC_color_RCI_002.tif	0 pairs			
Moving Image 5: BMC_color_RCI_003.tif	10 pairs	RMSE: 5 pixels		
Moving Image 6: BMC_color_RCI_004.tif	9 pairs	RMSE: 5 pixels	RMSE: 3 pixels	Saved!
Moving Image 7: BMC_color_RCI_004_flip.tif	11 pairs	RMSE: 4 pixels		Saved!

Navigate