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Protocol status: Working

Multiplex IHC Image Processing V0.2 V.2

Version 1 is forked from [HTAN Multiplex IHC Image Cytometry V0.2](#)

Shamilene

Sivagnanam^{1,2},

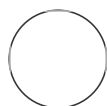
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NCIHTAN



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ABSTRACT

Understanding immune complexity within the tumor microenvironment provides valuable insight to tumor-immune composition, spatial interactions, and the immune system's reaction to therapy allowing us to better understand response and resistance at the interface of the tumor and host.

Multiplex IHC allows for the simultaneous detection of multiple Ab targets in the same tissue section using published methods previously described^{1,2,3}. This protocol contains detailed instructions on how to prepare files and run the image processing pipeline for mIHC SVS images in a GUI or command line, making it accessible to biologist and labs without computational specialty.

1. Tsujikawa, Takahiro, et al. "Quantitative multiplex immunohistochemistry reveals myeloid-inflamed tumor-immune complexity associated with poor prognosis." *Cell reports* 19.1 (2017): 203-217
2. Banik, Grace, et al. "High-dimensional multiplexed immunohistochemical characterization of immune contexture in human cancers." *Methods in enzymology* 635 (2020): 1-20.
3. Liudahl SM, Betts CB, Sivagnanam S, et al. "Leukocyte Heterogeneity in Pancreatic Ductal Adenocarcinoma: Phenotypic and Spatial Features Associated with Clinical Outcome." *Cancer Discov.* 2021;11(8):2014-2031. doi:10.1158/2159-8290.CD-20-0841

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GUIDELINES

This protocol is specifically for chromogenic multiplexIHC image processing using images scanned with Leica, Aperio ScanScope in the .SVS format.

BEFORE START INSTRUCTIONS

Make sure you have access to the following commercial softwares:

- Leica, Aperio ImageScope (free, Windows only)

Software	
ImageScope	NAME
Leica, Aperio	DEVELOPER

- Matlab R2018b or R2019b (license required)

Software	
MatLab	NAME

Additional toolboxes required: Computer Vision Toolbox

File prep and Folder Structure

1

FOLDER AND FILE NAMING CONVENTION

Make sure folder and file naming convention is consistent throughout the entire study. Filenaming convention should be used when saving images during acquisition.

Note

NAMING SLIDE FOLDERS AND IMAGE FILES

Each slide folder in a cohort batch should maintain some consistent format of letters and numbers:

Example slide folder name: 1 letter, 6 numbers

H342253

H342259

Each newly scanned image from one slide should be named with the following file naming convention at acquisition:

Initials_StudyID_UniqueSlideID_PanelName_CycleRound_Marker.SVS

Example file name:

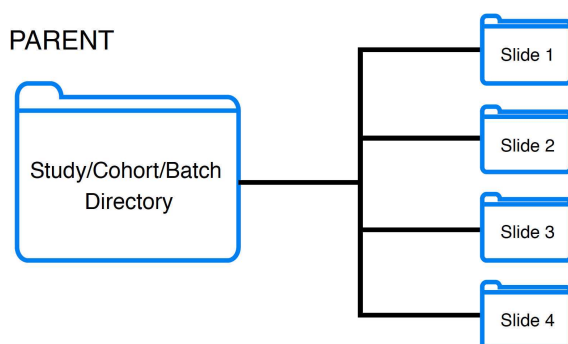
GG_HTAN1_H342253_D18_C01R1_PD1.svs

GG_HTAN1_H342253_D18_C01R2_PDL1.svs

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SET UP FOLDER STRUCTURE

The pipeline is optimal for batches of slides that run from a 'Parent' folder, that is the study or batch directory.



Folder structure for a cohort of slide images.

The study folder structure should be organized as the cohort/study/batch of slides as the parent folder, with each slide within the study containing all marker files with no duplicate scans of markers.

There should be an initial hematoxylin, a second hematoxylin after antigen retrieval, and a third hematoxylin at the end of the panel - see mIHC wet lab protocol for details:

Below is an example of the folder structure of a study called HTAN1 that contains 1 slide (H342253), 17 marker images, and 3 hematoxylin stained images.

Note

Example study folder structure:

```
|— HTAN1 (Parent Study)
| |— H342253 (Slide 1)
| | |— GG_HTAN1_H342253_D18_C00R1_HEM.svs
| | |— GG_HTAN1_H342253_D18_C01R1_PD1.svs
| | |— GG_HTAN1_H342253_D18_C01R2_PDL1.svs
| | |— GG_HTAN1_H342253_D18_C01R3_HEM.svs
| | |— GG_HTAN1_H342253_D18_C02R1_HLADPDQDR.svs
| | |— GG_HTAN1_H342253_D18_C02R2_DCLAMP.svs
| | |— GG_HTAN1_H342253_D18_C03R1_CD3.svs
| | |— GG_HTAN1_H342253_D18_C03R2_CD45.svs
| | |— GG_HTAN1_H342253_D18_C04R1_CD8.svs
| | |— GG_HTAN1_H342253_D18_C05R1_GRZB.svs
| | |— GG_HTAN1_H342253_D18_C05R2_CD20.svs
| | |— GG_HTAN1_H342253_D18_C06R1_CD68.svs
| | |— GG_HTAN1_H342253_D18_C07R1_CD66B.svs
| | |— GG_HTAN1_H342253_D18_C07R2_CD11C.svs
| | |— GG_HTAN1_H342253_D18_C08R1_CD163-001.svs
| | |— GG_HTAN1_H342253_D18_C09R1_EOMES.svs
| | |— GG_HTAN1_H342253_D18_C10R1_FOXP3.svs
| | |— GG_HTAN1_H342253_D18_C11R1_KI67.svs
| | |— GG_HTAN1_H342253_D18_C11R2_PANCK.svs
| | |— GG_HTAN1_H342253_D18_C11R3_HEM.svs
```

Get Code and Required Software

DOWNLOAD SOFTWARE AND CODE

Download or clone the MultiplexIHC_Code.zip file that contains all the functions and code for image processing. Save and unzip this folder somewhere other than your Downloads directory.

`git@github.com:Sivoyer/MultiplexIHC_Code.git`

In addition to this code, you will also need the following software installed:

Registration is performed in Matlab R2018b or higher:

Software	
MatLab	NAME

Additional toolboxes required: Computer Vision Toolbox

Color Deconvolution and Nuclei Segmentation is performed in FIJI:

Software	
FIJI (FIJI Is Just ImageJ)	NAME

Additional files required:  RGB_to_CMYK.class

To install this .class file, drag and drop into FIJI bar, then close and restart the program.

Single Cell Quantification is performed in Cell Profiler 3:

Software	
Cell Profiler	NAME

PIPELINE OVERVIEW

Once file names and folder structure is properly formatted for mIHC image processing, batch analysis can be performed. For every slide, multiple regions can be selected, avoiding regions of tissue deformation, necrosis, or other artifact or trouble tissue regions.

The image processing workflow utilizes steps commonly found in biological image analysis that have been optimized for brightfield RGB multiplexIHC images with AEC chromogen and hematoxylin. Although image processing can be completely automated, it is highly recommended that each step is checked to ensure data is as expected and is of high enough quality to continue processing. The modular steps allow for QC and adjustments at each major step.

Brief Description of Image Processing Workflow

Each region is registered to the final stained hematoxylin image from the same area, aligning each image such that major shifts due to repetitive scanning are adjusted for. Color deconvolution separates the AEC conjugated Ab signal from background. Single cell nuclei segmentation identifies cell areas using hematoxylin stain. Each identified cell is measured for mean intensity of every signal extracted marker.

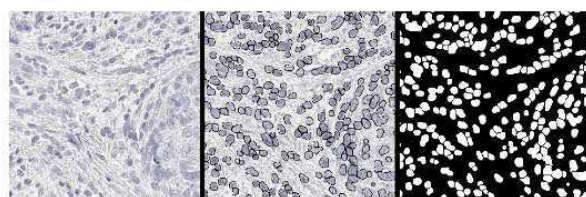
Multiplex IHC Image Processing

Each region is registered to all other stained images. Nuclei segmentation identifies cells, and color deconvolution separates AEC signal from background.

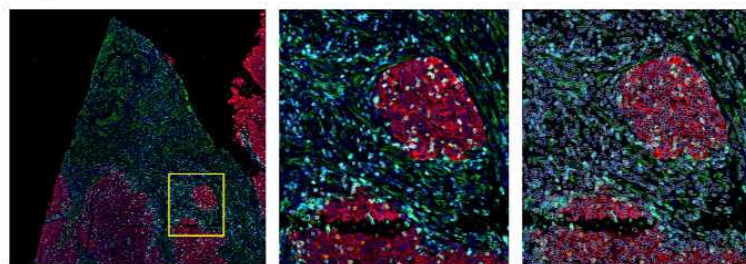
Region Selection → Registration → AEC color deconvolution



Single cell nuclei segmentation



Single cell measurements



Cell Number	Location		Mean Intensity			
	X	Y	Nuclei	CD45	CD3	CD68
1	98	39	0.00	0.00	0.00	0.54
2	122	45	0.24	0.22	0.22	0.00
3	78	33	0.14	0.12	0.12	0.00
4	137	46	0.17	0.06	0.06	0.02
5	123	41	0.01	0.14	0.14	0.00
6	121	46	0.13	0.18	0.18	0.00
7	228	59	0.01	0.02	0.02	0.42

Overview of image processing workflow

Region of Interest Selection

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SELECT REGIONS OF INTEREST

Regions are annotated in Aperio ImageScope, available for Windows only.

Software	
ImageScope	NAME
Leica, Aperio	DEVELOPER

Annotating Regions on final hematoxylin

Regions of interest are annotated on the last hematoxylin image to ensure that only cells maintained throughout all staining cycles and rounds are evaluated in downstream analysis.

Tissue areas that are lost, deformed, folded, or of poor quality should be excluded from analysis.

Note

DRAWING REGIONS IN IMAGESCOPE

1. Open the last cycle hematoxylin image in ImageScope.

Example: GG_HTAN1_H342259_D18_C11R3_HEM.svs

2. Select the rectangle tool for annotating boxes.

3. Draw boxes on areas of tissue that are of good quality (no folds, tissue loss, tissue deformation)

- Box sizes can be uniformly sized in settings, but only square/rectangle regions accepted.

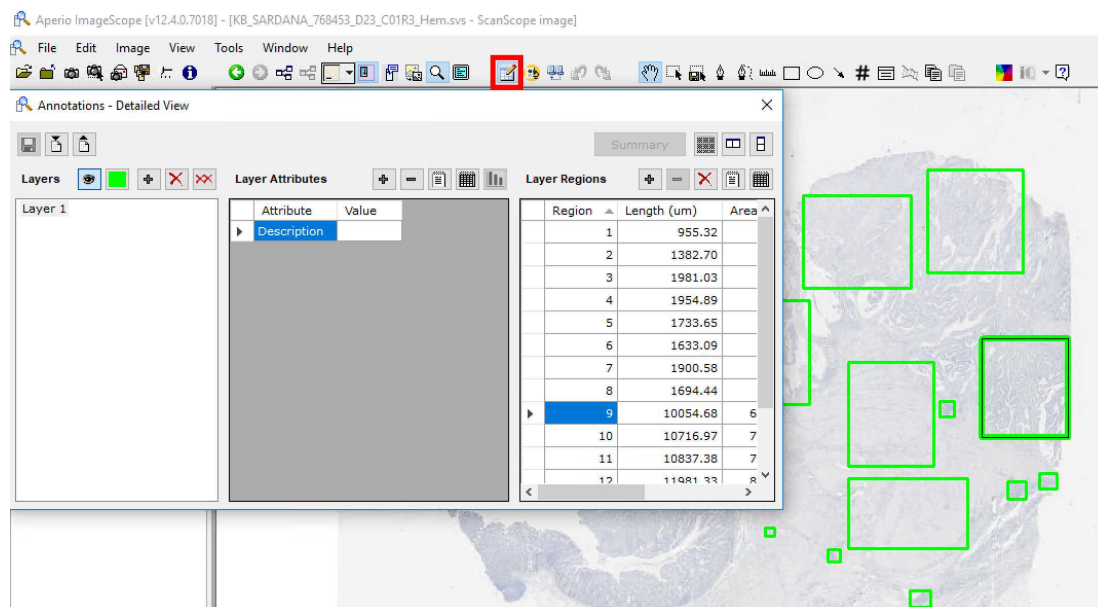
- Boxes should consist of tissue area with $\leq \sim 65000$ cells

- (constraint of ROI cell classification downstream if using FCS Express Image Cytometry)

4. When ROIs have been annotated, you can view the list of them on the Annotations - Detailed View in ImageScope

For more instructions on using Aperio ImageScope please see the manual:

 ImageScopeUserGuide.pdf



Annotations - Detailed View shows region annotations

5. Close the SVS file and you will be prompted to save annotations. Select yes, and ImageScope will save an XML with the same filename as the image it was drawn on (last hem). **Do not rename or move this file out of the slide folder containing the SVS images.**

Workflow Options

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Pipeline Run Options

This can be run using the software interfaces (code free), or by terminal. Instructions for both methods are listed below:

Step 6 includes a Step case.

Run mIHC Image Processing via Command Line

Run mIHC Image Processing via Software Interface

Image Registration

step case

Run mIHC Image Processing via Command Line

MacOS

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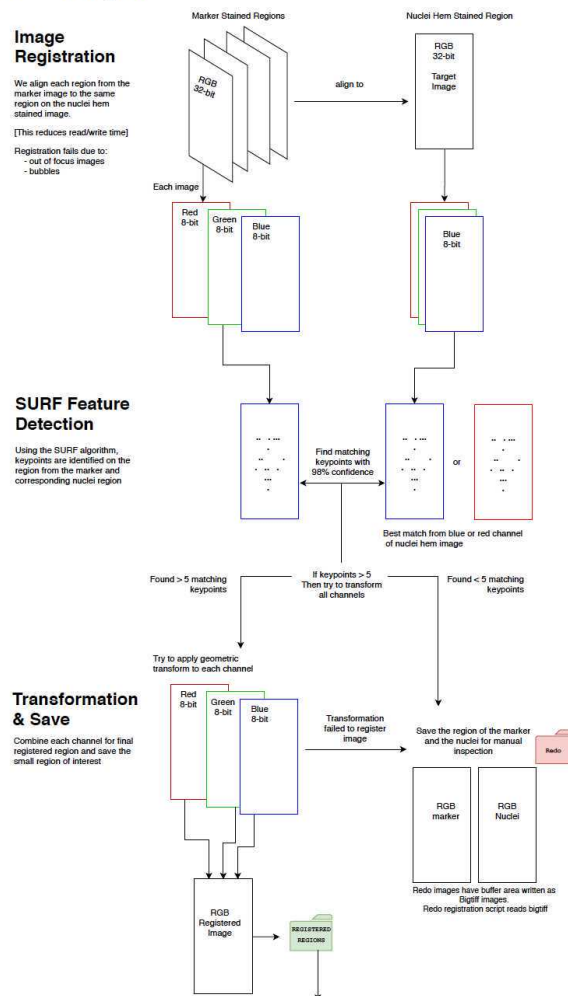
IMAGE REGISTRATION

How registration works:

Image registration is performed in Matlab. Each marker image is registered to the last cycle target hematoxylin image. Keypoints are identified on the blue channel of the hematoxylin image and the blue channel of the AEC marker image.

If not enough matching keypoints are found, other single RGB channels are checked for more keypoints, and the channels with the highest strongest matching indexed keypoints are used. A similarity based geometric transform is generated and applied to each channel of the marker image and merged. The registered RGB image is saved as a TIFF inside an automatically generated 'Registered_Regions' folder created in the slide/region folder.

For Each Region:



Workflow of image registration for each region of an image.

Command

code template to run matlab registration from linux mac on command line (macOS High Sierra 10.13.6)

```
"</path/to/matlab>" -nodesktop -nosplash -r "(cd('</path/to/matlab/code-dir>');run_mIHC_registration('</path/to/Parent-dir>');exit"
```

- Replace *<path/to/matlab>* with the path to your matlab application. For macOS, it is likely: */Applications/MATLAB_R2018b.app/bin/matlab*
- Replace *<path/to/matlab/code-dir>* with the path to the location where you saved the MultiplexIHC_RegistrationCode directory.
- Replace *<path/to/Parent-dir>* with the path to your Parent directory containing all slides to be registered.

Example:

```
"/Applications/MATLAB_R2018b.app/bin/matlab" -nodesktop -nosplash -r  
"cd('/Users/sivagnan/MultiplexIHC_Code/CommandLine/CL_Registration')  
;run_mIHC_registration('/Users/sivagnan/PROJECTS/Cohort1');exit;"
```

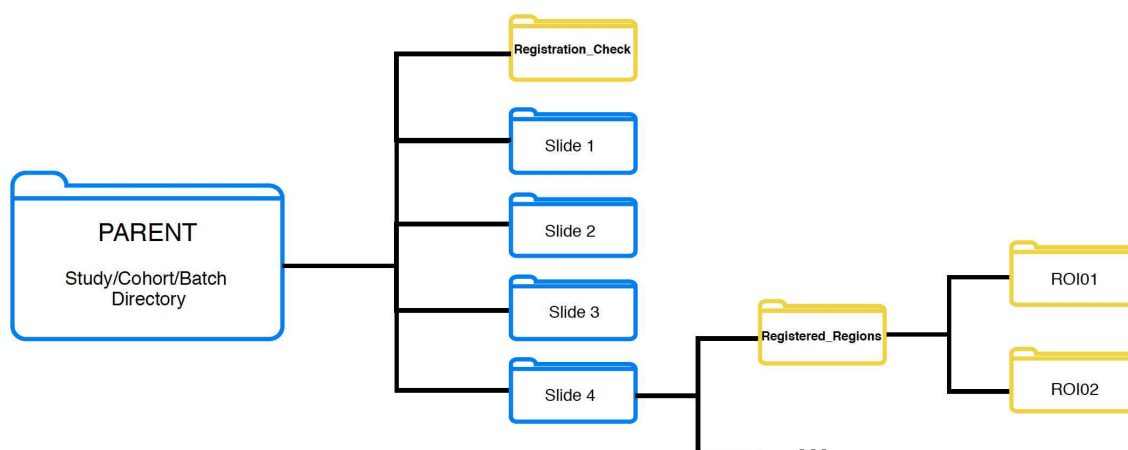
Image Registration Quality Control

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REGISTRATION QC

When registration is complete, you will see a Registered_Regions directory created inside each slide folder with an ROI folder for each region box annotation. Each ROIxx folder contains all marker images for that region with reg_ prepended to the filename.

A new folder called '**Registration_Check**' will be created in the Parent directory that contains low resolution images of all registered regions for every slide in the batch. Each low res image will have '**regck_**' prepended to the filename. This image set can be used to quickly check the images for gross misregistration or images that were false registered (passed but should have failed). Regions or stains that did not adequately register well enough for quantification due to tissue deformation or loss are removed from further analysis.



Folder structure after registration has completed. Folders in yellow are automatically generated during registration

If registration failed for a marker or region, you will see a 'Redo_ROI' folder inside the slide folder containing the failed marker region and the hematoxylin region that failed registration.

Images that failed registration can be excluded from further downstream analysis, or manually registered. See Troubleshooting guide ### for how to register images - 'Manual Registration' XXX.

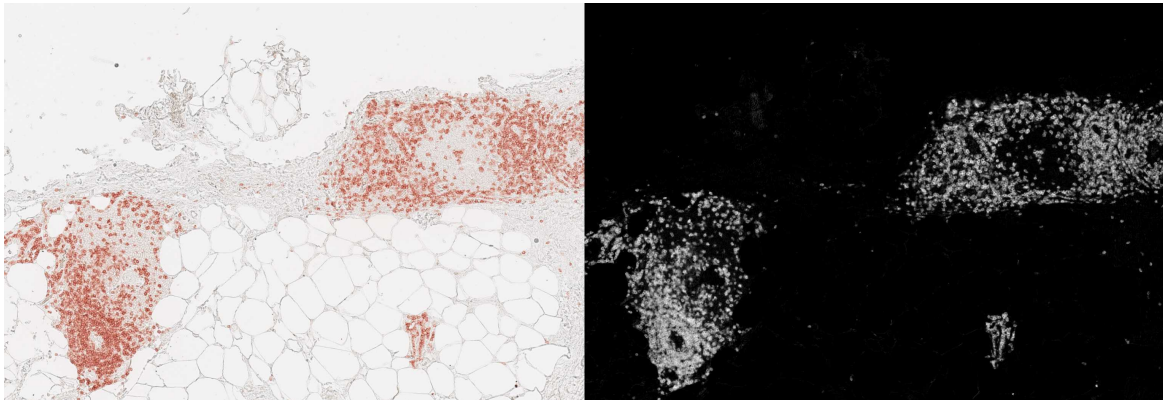
Signal Extraction

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AEC COLOR DECONVOLUTION

Color deconvolution separates the AEC signal from the tissue background to quantify signal.

AEC signal is extracted using an RGB to CMYK conversion which utilizes a maximum gray component replacement to subtract the lowest gray level from all channels. AEC chromogen signal is separated into the 'Y' and 'M' channels and the 8-bit image channels are added together. Pixel results greater than 255 are set to 255. The middle 90% of pixels are rescaled to 0-255 (Pham et al. 2007).



Left: Raw image with AEC stain for CD3

Right: Signal extracted CD3 signal post color deconvolution

Command

code template to run FIJI macros from mac on command line (MacOS HighSierra 10.13.6)

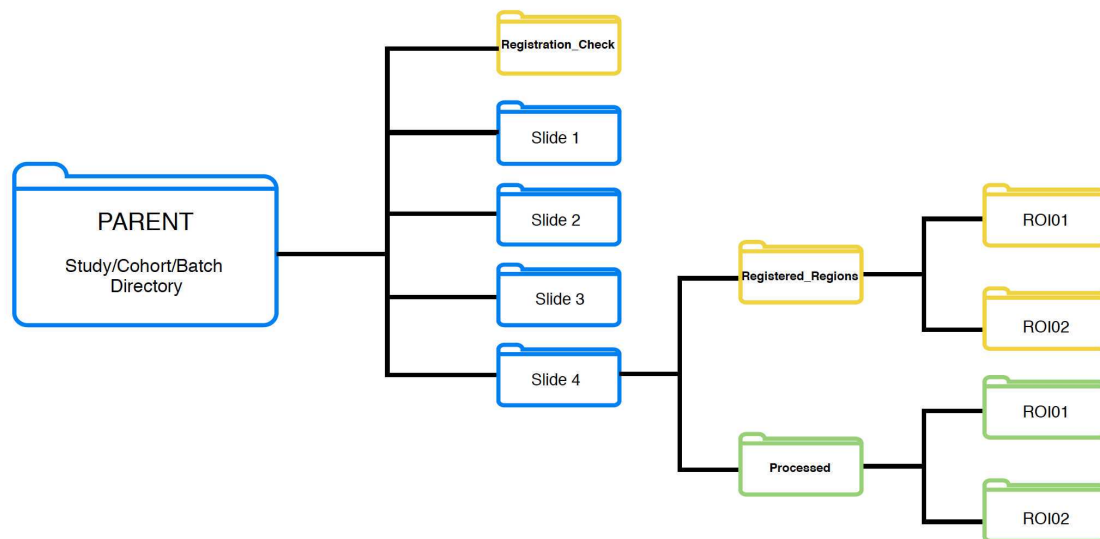
```
Java -jar </path/to/ij-xx.jar> -ijpath </path/to/Fiji.app> -batch </path/to/AEC_CMYK_ColorDecon.txt> </path/to/Parent-dir/>
```

EXAMPLE:

```
Java -jar /Applications/Fiji.app/jars/ij-1.52p.jar -ijpath  
/Applications/Fiji.app -batch  
/Users/sivagnan/MultiplexIHC_Code_072020/CommandLine/AEC_CMYK_ColorDecon.txt /Users/sivagnan/PROJECTS/Cohort1/
```

The script runs on the Parent directory and creates a directory in each slide called 'Processed'

with ROI folders inside; same folder structure as 'Registered_Regions'. Each generated image will have 'V_' prepended to the filename.



Folder structure after registration and color deconvolution. Yellow folders created during registration. Green folders created during color deconvolution.

Segmentation

10 NUCLEI SEGMENTATION

Segmentation is performed using the StarDist 2D_versatile_fluo algorithm implemented in FIJI python:

- [stardist/stardist: StarDist - Object Detection with Star-convex Shapes \(github.com\)](#)

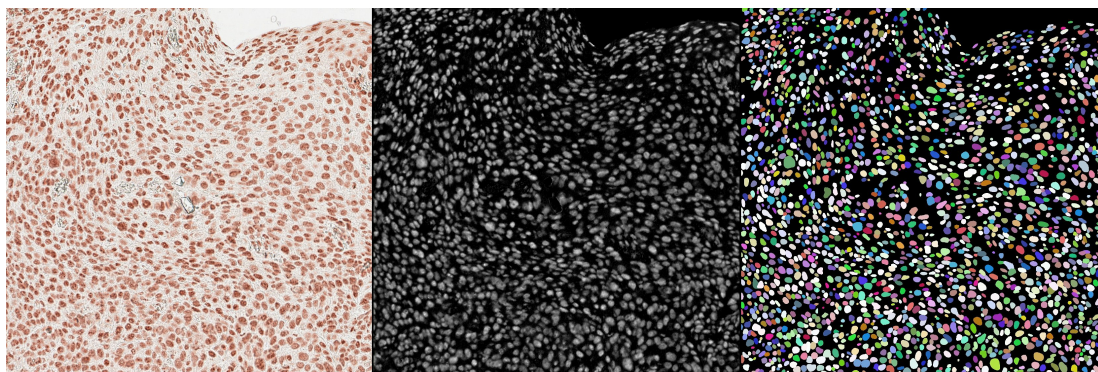
(Martin Weigert, Uwe Schmidt, Robert Haase, Ko Sugawara, and Gene Myers.

[Star-convex Polyhedra for 3D Object Detection and Segmentation in Microscopy.](#)

The IEEE Winter Conference on Applications of Computer Vision (WACV), Snowmass Village, Colorado, March 2020)

Segmentation can be performed on any single channel nuclear stain, preferably from the end of the staining panel to ensure only cells maintained throughout all staining cycles are evaluated downstream. If using a nuclear stain early in the panel, tissue and cells lost during the staining process can result in false negatives. We have optimized an antibody cocktail of H3+Nuclear Antigen to robustly identify single cell nuclei and this is recommended for segmentation in this

pipeline.



Left to right: raw IHC image, signal extracted image, labeled cell objects

BatchStardistSegmentation_H3NUCA.py

The script runs on the Parent directory and creates a labeled image for each ROI inside the Processed folder with a prefix, 'label_', based on the nuclei image named with 'V_H3NUCA_*'. This can be modified to identify any nuclei image name.

Single Cell Measurements

11 SINGLE CELL MEASUREMENTS

Single cell measurements are performed in Cell Profiler. Although there are many methods to measure mean intensity values for masked objects, this workflow utilizes Cell Profiler due to the output compatibility with FCS Express Image Cytometry for single cell classification. Measurements necessary for single cell gating are mean intensity for each marker, object area, object index, and object centroid X,Y location coordinates.

There are specific nomenclature that is required in order for the cell profiler output file to work properly in FCS Express Image Cytometry.

See the attached document on how to use the template Cell Profiler project, which can be found in the MultiplexIHC_Code directory.

 CellProfiler_TemplateInstructions_V0.2.pdf

Command

python command to run a cell profiler batch.h5 file on linux for batch processing (Linux)

```
./python -m cellprofiler -p <Default_Output_Folder_path>/Batch_data.h5 -c -r -b -f <first_image_set_number> -l <last_image_set_number>
```

Example of running 50 image sets from the file Batch_data.h5 located in the project folder, Cohort1, on Linux:

```
./python -m cellprofiler -p  
/Users/sivagnan/PROJECTS/Cohort1/Batch_data.h5 -c -r -b -f 1 -l 50
```

Command

running batch image sets using a batch.h5 file on Mac (High Sierra 10.13.6)

```
/Applications/CellProfiler-3.1.5.app/Contents/MacOS/cp -c -r -p <Default_Output_Folder_path>/Batch_data.h5 -f <first_image_set_number> -l <last_image_set_number>
```

Example of running 50 image sets from the file Batch_data.h5 located in the project folder, Cohort1 on MacOS (change CellProfiler version to your version):

```
/Applications/CellProfiler-3.1.5.app/Contents/MacOS/cp -c -r -p  
/Users/sivagnan/PROJECTS/Cohort1/Batch_data.h5 -f 1 -l 50
```

This command submits the batch file to CellProfiler and specifies that CellProfiler run in a batch mode without its user interface to process the pipeline. This run can be modified by using additional options to CellProfiler that specify the following:

- -p <Default_Output_Folder_path>/Batch_data.h5: The location of the batch file, where

<Default_Output_Folder_path> is the output folder path as seen by the cluster computer.

- -c: Run “headless”, i.e., without the GUI
- -r: Run the pipeline specified on startup, which is contained in the batch file.
- -b: Do not build extensions, since by this point, they should already be built.
- -f <first_image_set_number>: Start processing with the image set specified, <first_image_set_number>
- -l <last_image_set_number>: Finish processing with the image set specified, <last_image_set_number>

Visualization stack (optional)

12 CONVERT TO OME.TIFF

The 'V_' prepended images are your registered, signal extracted, single channel 8-bit final image. Each set of images within an ROI can be converted into a single ome.tif using bioformats to create Level 2 data.