



















## Map of Directories:









The following document outlines the possible output of directories created when following the step-by-step protocols of “Pre-Processing,” “Training,” “Validation,” and “Analysis.” Output may slightly vary depending on your specific research needs, but the general format should be consistent.

### Pre-Processing











-  **Project File** (this file contains all of the data associated with a set of slides that are stained for specific purposes)
  -  **Originals** (all of the .tiff image files from fluorescence slide scans, these are multichannel image files of entire slides)
  -  **Separate Slide to Sections Project** (all information to transform individual slides of multichannel tissue cross sections to single channel tissue cross sections)
    -  **QuPath Separate Sections** (project folder that will extract tissue sections from slide as multichannel .tiff files)
    -  **Extracted Multichannel Whole Section** (multichannel .tiff files that describe the location of the tissue section on the slide)
  -  **Extracted\_Decoded\_Split\_Analyzed** (contain all the data associated with stains and experimental condition)
    -  **QCMosaic Pre-Segmentation** (this contains all of the consolidated image files into one mosaic based on stain)
      -  **Stain1**
      -  **Stain2**
      -  **Stain3**
    -  **Stain1** (all of the single channel .tiff files of tissue sections associated with a stain)
    -  **Stain2**
    -  **Stain3**






### Training

-  **[Stain]**
  -  **Original Images ROIs** (all of the cropped images that will be used for training—free of artifacts-- will be contained within this folder)
  -  **Original Images Full Size** (full size images for the training project will be placed here)
  -  **Original Images Full Size\_Segmentation\_[timestamp]**
    -  **Overlays** (contains U-Net assisted segmentation annotations overlayed onto the original single channel .tiff files of the original images full size)

-  **Segmentations** (contains U-Net assisted segmentation annotations while subtracting all background imaging)
-  **[Stain]-Extracted Training Images (ALL)** (this is the output folder created when running macro to select regions of interest within a full-size image)
-  **ROIs (Cropped Images-ALL)** (this is the output folder created when running macro to select regions of interest within a full-size image—this will be the same or very similar to the content of “Original Images ROIs”)
-  **ROI Locations** (the location of the cropped image ROI on the full size image)
-  **Qupath [Stain] Training** (this contains all of the project information for annotating images for training)
-  **Annotations (from QuPath)** (contains all of the annotation data exported from “QuPath [Stain] Training”)
-  **Annotated Images for Training** (the annotation data overlayed onto the “Original Images ROIs”)
-  **Training Output** (data files association with stain detection)

## Validation

-  **[Stain]** (this will contain all the data to validate U-Net Segmentation under one stain)
  -  **Validation Analysis** (this contains all personal data from individual lab staff that are annotating images)
    -  **Image Overlays (REF vs SEG)** (images of overlays between human reference annotations and the segmentation output)
    -  **Point Data Files** (“ .csv” files contains the coordinate locations and reference values between human reference annotations and segmentations)
  -  **Reference Annotations** (**nuclear**: contain the reference annotations using multipoints; **cytoplasmic**: annotations exported from QuPath project)
  -  **Reference Annotations QuPath Project** (**this is only generated when using cytoplasmic stains**: QuPath project with reference annotations)
  -  **Original Images ROIs** (all of the cropped images selected at random that will be used for analysis will be contained within this folder)
  -  **Segmented Images ROIs** (all of the cropped \*segmented\* images selected at random that will be used for analysis will be contained within this folder)
  -  **Original Images Full Size** (randomly selected full size images for the validation project will be placed here)
  -  **Segmented Images Full Size** (the corresponding segmented files that match the “Original Images Full Size” files will be here)














-  **Segmented Images ROIs with Multipoints** (all of the cropped \*segmented\* images that have multipoints added one will be within this folder)
-  **[Stain]\_Extracted Validation Images (ALL)** (this is the output folder created when running macro to select regions of interest within a full-size image)
  -  **ROIs (Cropped Images-ALL)** (there should be five cropped image ROIs per full size image here—this is where you will randomly select one cropped ROI from to copy and paste into “Original Images ROIs”)
  -  **ROI Locations** (the location of the cropped image ROI on the full size image)
-  **Selected ROI Locations**(the coordinate locations of the randomly selected original images cropped for validation)






















## Analysis

*Analysis expands the folders that were generated in the “Extracted\_Decoded\_Split\_Analyzed” folder during Preprocessing.*

*Folders generated in Preprocessing are in gray italics*

**Folder generated in Analysis are in black**

-  *Extracted\_Decoded\_Split\_Analyzed* (contain all the data associated with stains and experimental condition)
  -  **Analysis** (contains summary tables that include the total area analyzed, the area of the segmentation, and area fraction of each stain)
  -  **Cortex Raw Borders (FULL SIZE)** (this contains the unrefined section that defines only the cortex-medulla border—all exported annotations from “QuPath Cortex Raw Borders” after adjusting for resolution)
  -  **CORTEX ONLY OUTLINES** (contains the refined section that encompasses only the cortex and separates out artifacts within tissue section)
  -  **LOW RES** (all low resolution files used to create “CORTEX ONLY OUTLINES”—these are not used within analysis)
    -  **Cortex Raw Borders (LOW RES)** (this contains the unrefined section that defines only the cortex-medulla border—all exported annotations from “QuPath Cortex Raw Borders” before adjusting for resolution)
    -  **[Stain1] (LOW Res)**
    -  **[Stain2] (LOW Res)**
    -  **Whole Section Outlines (LOW RES)** (all exported annotations from “QuPath Whole Section Outlines” before adjusting for resolution)
  -  **QCMosaic Pre-Segmentation** (this contains all of the consolidated image files into one mosaic based on stain)
    -  **Stain1**
    -  **Stain2**
    -  **Stain3**

-  **Stain4**
-  **QCMosaic Post-Segmentation** (this contains all of the consolidated segmented image files into one mosaic based on stain)
-  **Stain1** (all of the single channel .tiff files of tissue sections associated with a stain)
-  **Stain1\_Date\_Segmentation**
  -  **Overlays** (contains all of the U-Net assisted segmentation annotations overlayed onto the original single channel .tiff files of tissue sections)
  -  **Segmentation** (contains all of the U-Net assisted segmentation annotations while subtracting all background imaging)
-  **Stain2**
-  **Stain2\_Date\_Segmentation**
  -  **Overlays**
  -  **Segmentation**
-  **Stain3**
-  **Stain3\_Date\_Segmentation**
  -  **Overlays**
  -  **Segmentation**
-  **Stain4**
-  **Stain4\_Date\_Segmentation**
  -  **Overlays**
  -  **Segmentation**
-  **QuPath Whole Section Outlines** (project folder to create outlines of tissue section and exclusion of artifacts)
-  **QuPath Cortex Raw Borders** (project folder to encompass the cortex and define cortex-medulla border)
-  **Whole Section Outlines (FULL SIZE)** (all exported annotations from "QuPath Whole Section Outlines" after adjusting for *resolution*)

**Commented [MM1]:**

This is not part of the protocol for the Analysis Section but it is present in the mock project. Let me know if you want me to delete it or not.

**Commented [BJ(2R1):** Is there a way we could mark the folders here that we made in pre-processing to differentiate them from the ones made in analysis