
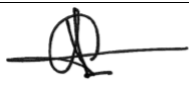
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Digital Image Analysis of Whole Slide Images			Page 1 of 9
Authors: Guray Akturk, MD; Sacha Gnjatic, PhD			SOP Number: HIMC-...
Approvals			
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1. Purpose

Scanning of pathology slides generates whole slide images and being able to analyze these images, either individually to establish density (per mm²) and/or percentage (out of all nucleated cells) of stained cells expressing a marker, or for a selected morphological feature is a challenge. Manual counting is feasible for an individual image with a small tissue but can quickly become overwhelming, pointing to the growing use of digital pathology solutions when multiple markers are involved. The following image analysis pipeline is based on an open-source software named QuPath.

2. Abbreviations

ROI: Region of Interest

RGB: Red Green Blue

H-DAB: Hematoxylin – Diaminobenzidine

TMA: Tissue Micro-Array

3. Safety Considerations / Personal Protective Equipment

n/a

4. Materials and Equipment

Desktop Workstation Computer

QuPath 0.1.2 Image Analysis Software

Whole Slide Images




5. Quality Control

Quality control is performed by reviewing the slide images and recorded image analysis data for the quality of cell segmentation and classifications by an experienced pathologist.

6. Method

6.1. Project Management

Regardless of organized image folders for each project, QuPath has a feature of project management feature to group images belong to a project together and save every single image analysis data in that project to work on them anytime needed. It creates data files with 'qpdata' extension for every image in a project to save image analysis

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data like annotations, cell segmentation, and phenotyping data. QuPath stores project folder in a different location than the image files.

6.1.1. Project is created with a specific name

6.1.2. Related images are imported into the project. QuPath provides several methods to import images like selecting files one by one or importing a selected folder of images altogether.

6.1.3. Metadata like antibody name, date and order of cycle are encoded in each image to sort them in different desired order.

6.2. Stain Vectors

Chromogenic immunohistochemistry images are basically RGB images per their background pixel values and three important stain vectors (hematoxylin, chromogen and residual) are used for immunostaining separation. Since QuPath uses color deconvolution for stain separation, stain vector values are extremely important for further steps of image analysis. QuPath has a very useful feature for estimating these values named 'Estimate Stain Vectors' for the purpose of improving stain separation.

6.2.1. Default stain vector type should be selected as Brightfield H-DAB



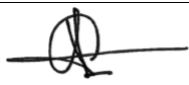
6.2.2. Representative tissue ROI is selected on tissue. This ROI must include balanced amount of positive and negative cells as well as blank (white) area. Moreover, it should not be a large region since it results in down sampling of pixel values and it should be avoided for best results.

6.2.3. 'Estimate Stain Vectors' feature is used for automatic estimation of stain vectors. Results are shown in 2D scatterplots belong to three vectors.

6.2.4. Scatterplot shows stain vector values by dots and two stain vectors per scatterplot. Most of the dots should fall ideally between two stain vectors in each scatterplot.

6.2.5. Quality control is made by visualizing each vector in Brightness/Contrast tool.

Note: If there are many images in a project that are acquired with a similar staining, one representative ROI can be used to estimate stain vectors in batch mode by using a script.

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6.3. Annotation

Annotations can be made in different ways according to the purpose of a project. It's needed for further steps of image analysis since analysis is performed on created annotations in the image.

6.3.1. Simple tissue detection: This tool can automatically recognize and annotate whole tissue on the slide.

6.3.2. Annotation tools: QuPath provides many basic annotation tools like rectangle, circle, line tool as well as more advanced ones like wand tool which detects sharp changes in tissue and annotate it by moving the pointer around the interested area.

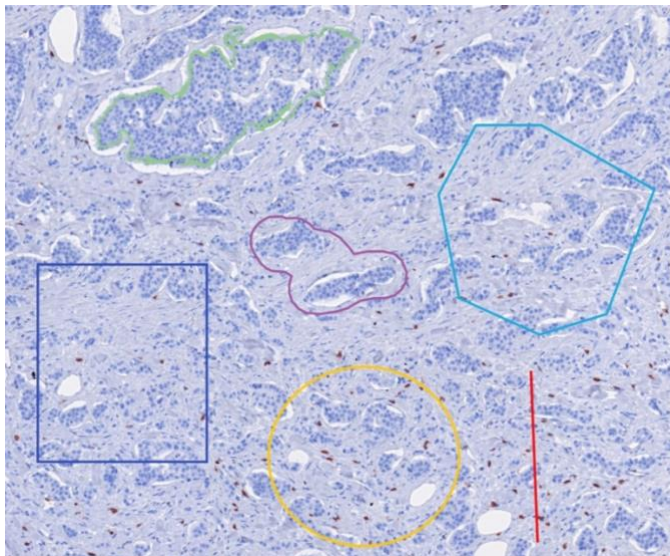


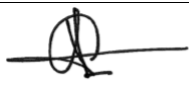


Figure 1: Different annotation tools provided by QuPath.

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6.3.3. TMA annotation: TMA cores can automatically be annotated by the TMA support of QuPath.

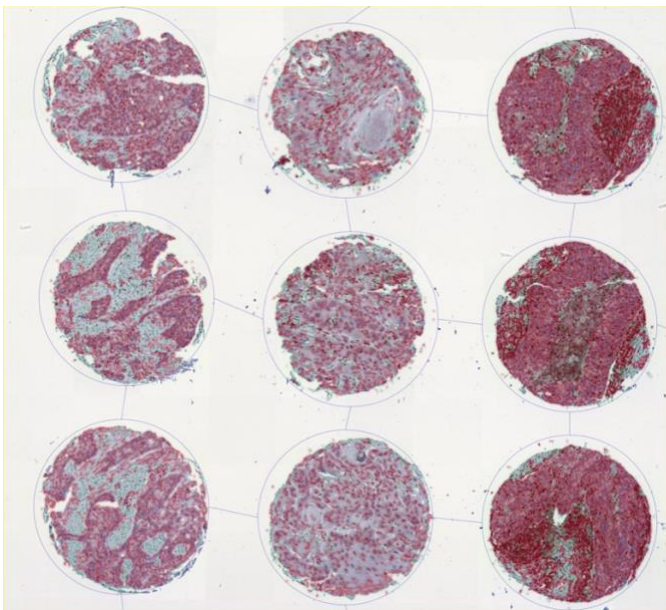


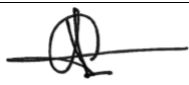


Figure 2: TMA annotation tool.

6.3.4. Annotation of specific tissue structures like tumor should be done by using the annotation tools mentioned above by a pathologist to prevent possible errors.

NOTE: Tumor annotation can be expanded inwards and outwards for a desired diameter to analyze tumor border and peritumoral regions.

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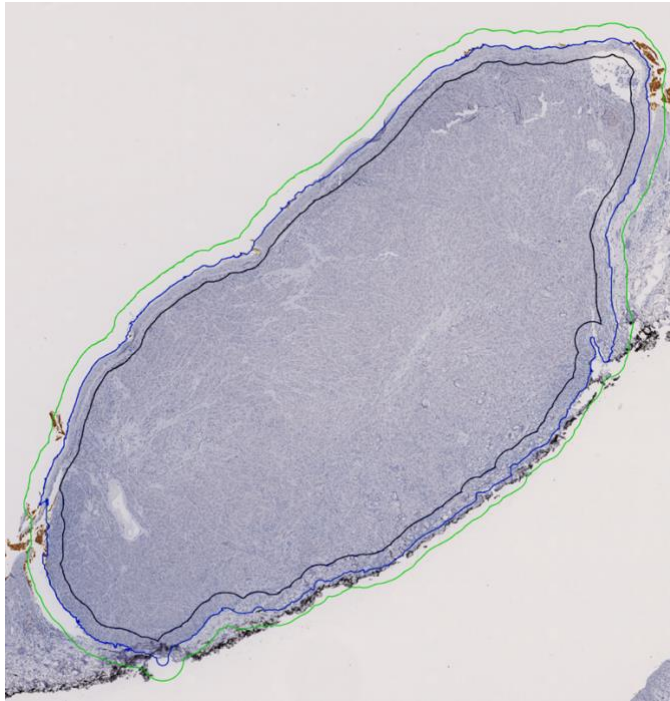


Figure 3: Expansion of tumor (malignant melanoma) annotation inwards and outwards



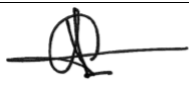
6.4. Cell Segmentation

Cell segmentation refers to detection of cells in an image. QuPath basically makes nuclear segmentation and expands the nuclear border of detection by a selected diameter to create a cytoplasmic zone.

6.4.1. Detection image type is set to either 'Hematoxylin OD' or 'Optical density sum'

6.4.2. Nuclear parameters are tweaked according to tissue type.

6.4.2.1. Background radius value estimates the background color and improves the cell segmentation. It makes this estimation pixel by pixel and propagates the information throughout the tile by using the background radius value. This works in images with a lot of texture in the background or images with background staining, however it's not helpful in all occasions so its use is optional.

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6.4.2.2. Median filter and Gaussian filter values are used to smooth the image to ease the minimal details inside the cellular compartments to segment cells properly and accurately. High values are especially helpful in segmenting large cells like CD68 positive histiocytes.

6.4.2.3. Minimum and maximum nuclear area values are tweaked for discarding small and large artifacts and set the segmentation algorithm just to segment real nuclei.

6.4.3. There are two different intensity parameters;

6.4.3.1. Threshold for selected detection image is one of the most important settings in cell segmentation and it basically sets an intensity cut off for detection of positive immune cells. Multiple cut off values can be set for multiple intensity levels. Threshold can also be used with morphological features to differentiate cells with various shapes like large anaplastic tumor cells and lymphocytes from each other.

6.4.3.2. Maximum background intensity value can be useful to get rid of artifacts by decreasing its value.

6.4.4. Cell expansion value sets the area of cytoplasmic region of segmented cells, however if cells are squeezed together in a dense tissue, adjacent cells automatically decrease the size of this expansion area and limits it in their neighborhood.

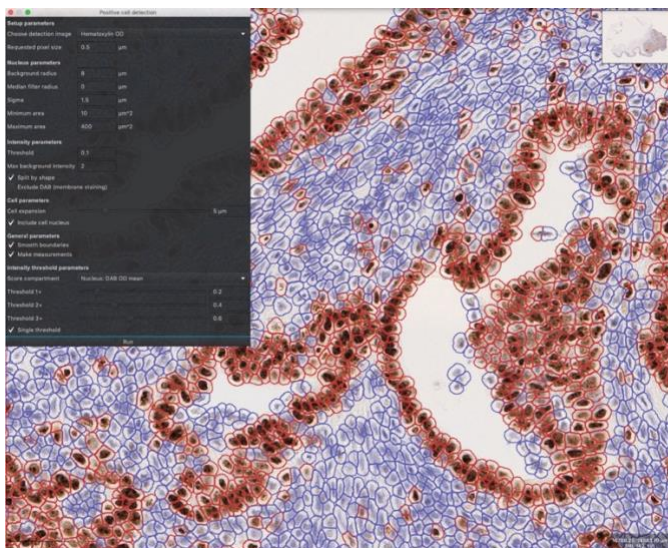


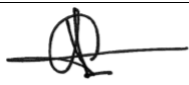


Figure 3: Cell segmentation and positive cell detection by using mean DAB color intensity.

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6.5. Phenotyping

Phenotyping can be performed by two different methods.

6.5.1. Thresholding

6.5.1.1. Measurement maps is used to set a cut off value for a desired feature.

6.5.1.2. Once cut off value is set, it is applied to the image to differentiate the cells for a selected feature like shape or intensity.

6.5.1.3. Data can be extracted from annotation measurements or detection measurements and it can be exported to an excel file.

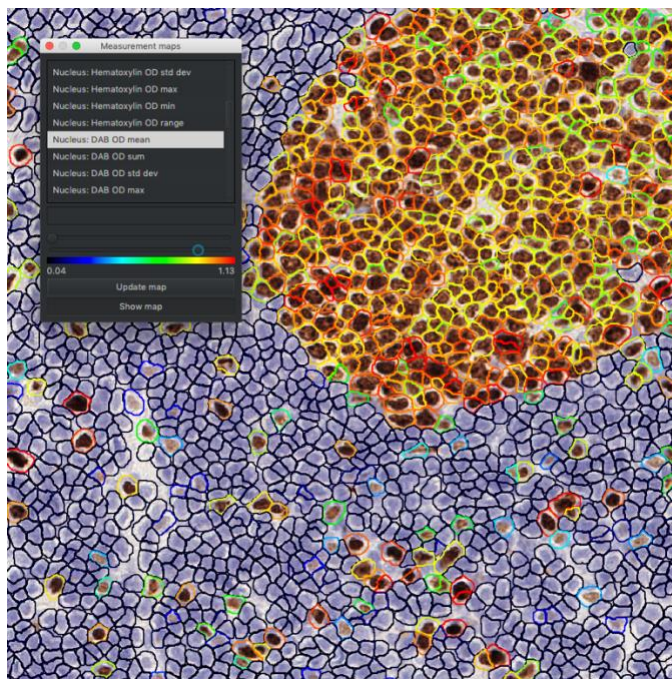





Figure 4: Measurement maps is used to review different cut off values for a selected feature. “Nucleus: DAB OD Mean” feature is used for detecting positive stained immune cells in this ROI and measurement maps helped with finding a cut off value which differentiates positive and negatively stained immune cells from each other.



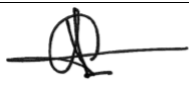
6.5.2. Machine Learning Based Classifier

Use of this method varies according to the purpose of analysis. If morphological classification is the interest, ‘add smoothed features’ option can be used to

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improve the classification. However, if the aim is just obtaining results for positive cell detection, smoothing process is usually not needed (except markers like cytokeratin which stains group of cells). Smoothing reduces the noise of measurement of shape features like nucleus/cell ratio. It takes weighted average of detection measurements of adjacent cells.

- 6.5.2.1. Smoothed features can be added according to the aim of the project.
- 6.5.2.2. Detection classifier is created by selecting a classifier type. Random forest method is the default classifier algorithm.
- 6.5.2.3. Desired features are selected from advanced options according to the aim of the project.
- 6.5.2.4. Cell/tissue type classes are created in annotations tab.
- 6.5.2.5. Different cell classes are annotated using annotation tools. Using dots for different classes is useful for detection of positive immune cells.
- 6.5.2.6. Classifier system will auto-update classification in annotations which include segmented cells during the annotation of different classes. Once the desired classification is achieved, classifier and training objects are saved separately.
- 6.5.2.7. Saved classifier can be applied to other images in the project file. Batch run of a saved classifier can be performed to save time, however review is always needed for quality control.
- 6.5.2.8. Training objects can be loaded any time on other images to continue training the classifier, if there is any inaccurate classification in other images in the project.

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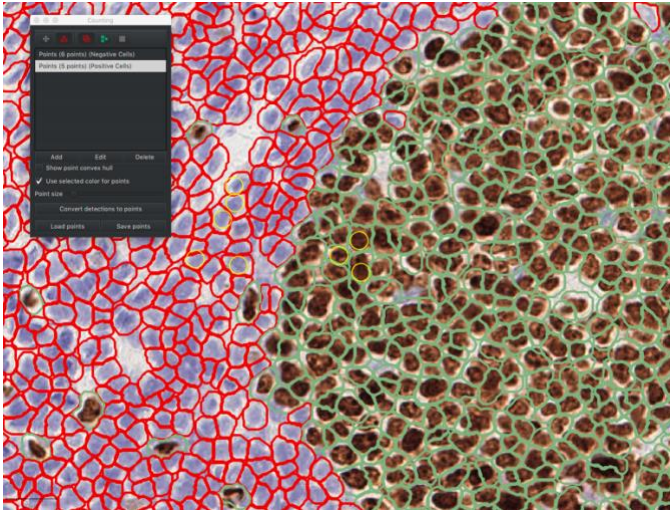


Figure 5: Random Forest algorithm classifies cells as positive and negative by using a combination of different DAB color intensity features here. Yellow circled cells are selected for training the algorithm.

6.6. Data Analysis

Final data can be extracted as annotation or detection measurements and exported as a data file.