

Supplementary File 1

1 **QuPath protocol for Ki67 IHC evaluation on core biopsy slides and on whole slides**

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6 **International Ki67 in Breast Cancer Working Group**

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8 **Task: Set up and Use of QuPath for Ki67 evaluation on 30 core biopsy -and 30 whole**
9 **section slides stained with Ki67 and visualized with DAB on Ventana platform in Breast**
10 **Cancer.**

11 Download [QuPath](#).

12 **Supported image formats:**

Recommended specification:

- 13 • Whole slide image formats:
 - 14 ○ Aperio (.svs, .tif)
 - 15 ○ Hamamatsu (.vms, .vmu, .ndpi)
 - 16 ○ Leica (.scn)
 - 17 ○ MIRAX (.mrxs)
 - 18 ○ Philips (.tiff)
 - 19 ○ Sakura (.svslide)
 - 20 ○ Trestle (.tif)
 - 21 ○ Ventana (.bif, .tif)
 - 22 ○ Generic tiled TIFF (.tif)
 - 23 ○ Perkin Elmer (.qptiff)
- 24 • ImageJ TIFF
- 25 • JPEG
- 26 • PNG

- Multi-core 64-bit processor & operating system (e.g. Intel i7)
- 12 GB RAM
- Discrete graphics card
- Solid state hard disk
- Lots of hard disk space to store images and data - may require more than 1 GB to store data related to a single slide

27 To create a project, choose *File/ Project../ Create new project*. You will then have to select an
28 empty folder in which the project will be made. You should create 2 separate projects: One
29 for the whole slide cases and one for the core biopsy cases.

30 To add the 30 slides to the current project, click *File/Project../Add images*, then use *Choose*
31 *files* to select the 30 slides, next click *Import*.




32 You can create an *images* folder inside your project folder, and copy (or move) your images
33 inside it. In this case, everything will be kept in the same folder - and continue to work, no
34 matter where you move the project folder.

35 The next time you want to open your project, choose *File/ Project../ Open project*, then go to
36 your project folder and select the *project.approj* file.

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37 Step 1: Annotation objects

38 Start with the slide 56_MIB1 for core biopsy cases and slide 4_MIB1 for whole section cases.


39 Annotate the whole tissue on each slide with the  polygon tool, the  wand tool or
40 the  brush tool (left click + dragging the mouse across the image.)

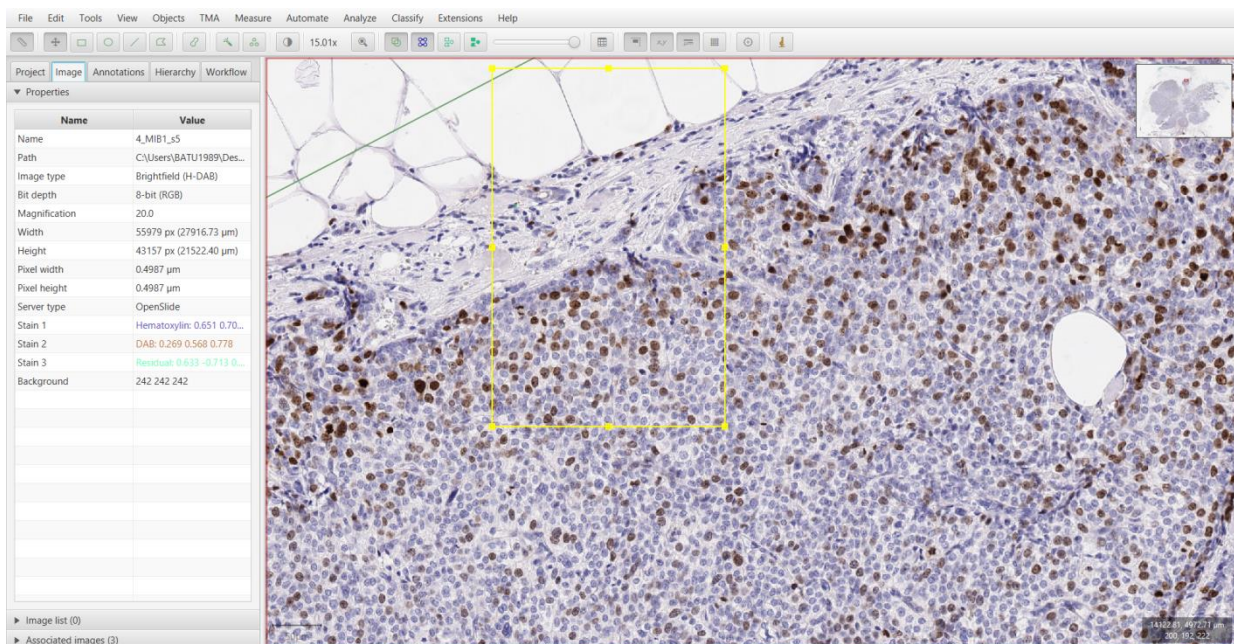
41 To delete an annotation, go to *Annotations* tab, select the annotation with left click then left
42 click on delete. Try to annotate the entire relevant tissue as close as you can. Avoid damaged
43 tissue at the edges of the specimens. If you see areas of normal tissue, DCIS or artifacts
44 without any considerable area of invasive tumor, you should avoid them when making the
45 large annotation. If you see larger number of normal epithelium, DCIS and artifacts (e.g.:
46 irrelevant DAB staining) within invasive tumor areas, please refer to Step 4, line 100-104.

47 Step 2: Estimate stain vectors

48 It is recommended to refine the stain estimates for each new image.

49 You should first find a representative region containing clear examples of the stains that you

50 want - along with an area of background. Draw an annotation with  rectangle tool
51 around a region containing examples of each stain and background (whitespace). You should
52 try to choose a small region because in case of a large region QuPath will have to
53 downsample it (by averaging adjacent pixels - which dilutes the useful information) to look
54 for the stains.



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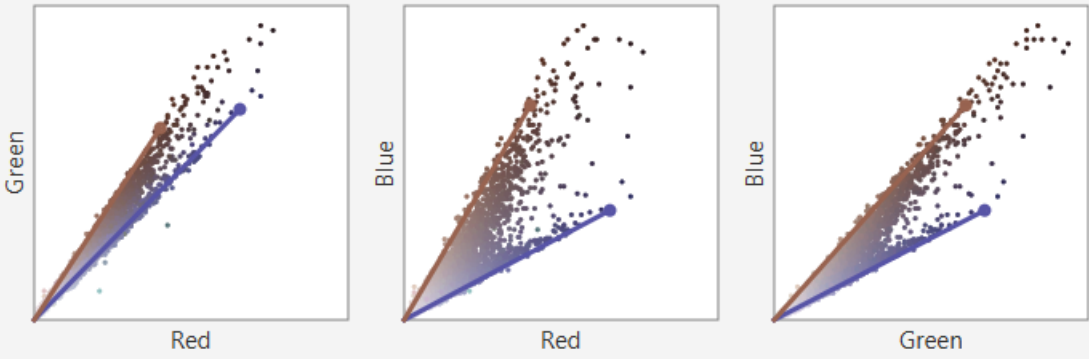
56 You should try to capture all staining intensities. If this is not possible in a small rectangle,
57 choose a high staining intensity region.

58 Then, go to *Analyze/Preprocessing/Estimate stain vectors*

59 If the background values in the drawn region do not match with the background values
60 currently being used, QuPath will ask you whether you want to update the stored values.
61 Assuming that the region you have drawn does contain a representative area of background,
62 you should click *Yes*.

63 Click on *Auto*, check the updated stain vectors then *OK*. Then, set a name for the updated
64 stain vectors.

Visual Stain Editor ×



▼ Stain vectors

Name	Original	Current	Angle
Hematoxylin	0.651 0.701 0.29	0.655 0.67 0.348	3.77
DAB	0.269 0.568 0.778	0.401 0.61 0.683	9.64
Residual	0.633 -0.713 0.302	0.591 -0.742 0.316	3.02

▼ Auto detect

Min channel OD

Max total OD

Ignore extrema %

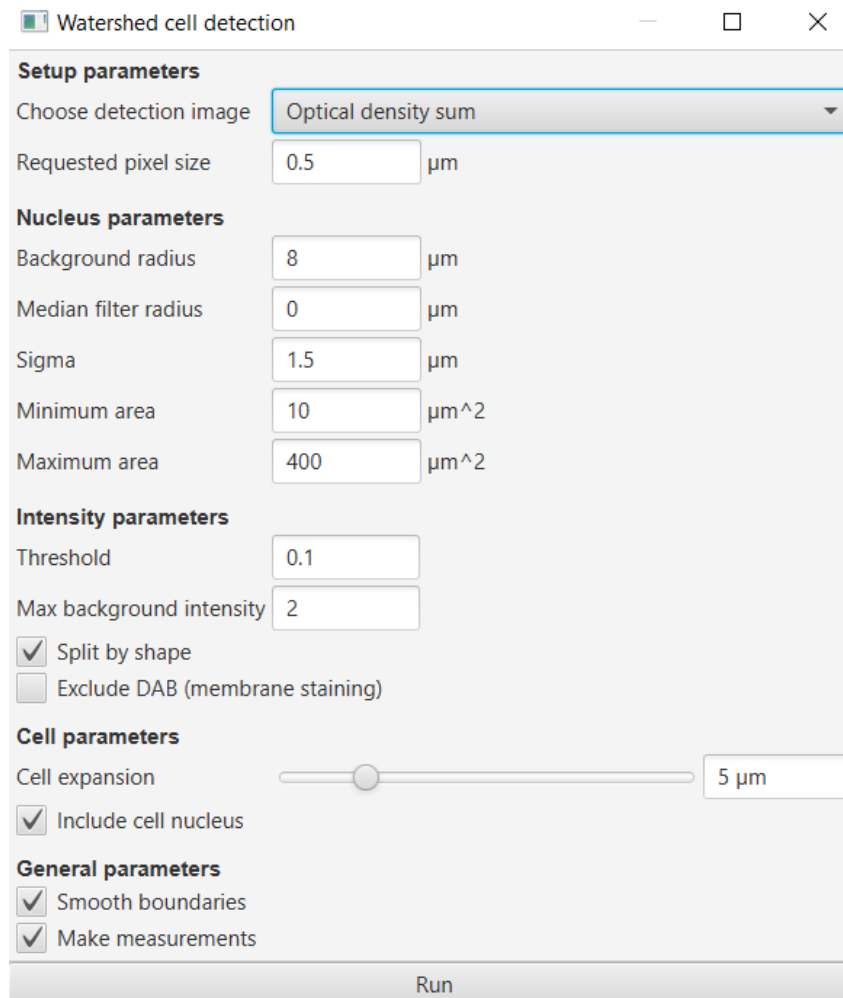
Exclude unrecognised colors (H&E only)

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66 Step 3: Cell detection

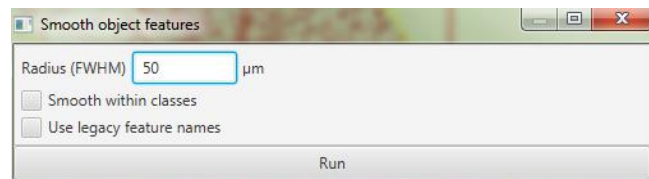
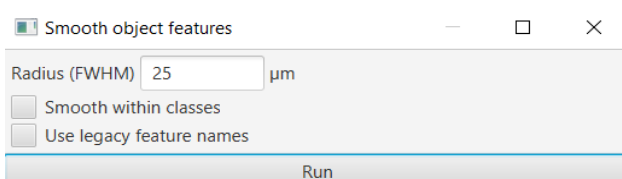
67 You should select the original, large annotation containing the whole tissue, then go to
68 *Analyze/Cell analysis/Cell detection*. This will bring up a dialog, where most of the options
69 relate to how the cells are detected. Choose *Optical density sum* (at “*Choose detection*
70 *image*”), other default values are good for Ki67 IHC in breast cancer. Click *Run*.



71

72 The use of optical density will result the detection of stronger background, blood cells. To
73 handle this, refer to Step 4, line 97-98.

74 To help QuPath perform an accurate classification it is useful to supplement the existing
75 measurements of individual cells with some additional features that take into consideration
76 more contextual information. Thus, run the *Analyze/ Calculate features/Add smoothed feature*
77 command at 25 μm and 50μm.



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78 Step 4: Create classification


79 You should build 2 separate classifiers: One for core biopsy slides and one for whole slides

80 To set up a classifier for core biopsy (CB) slides use the slide 56 MIB1.

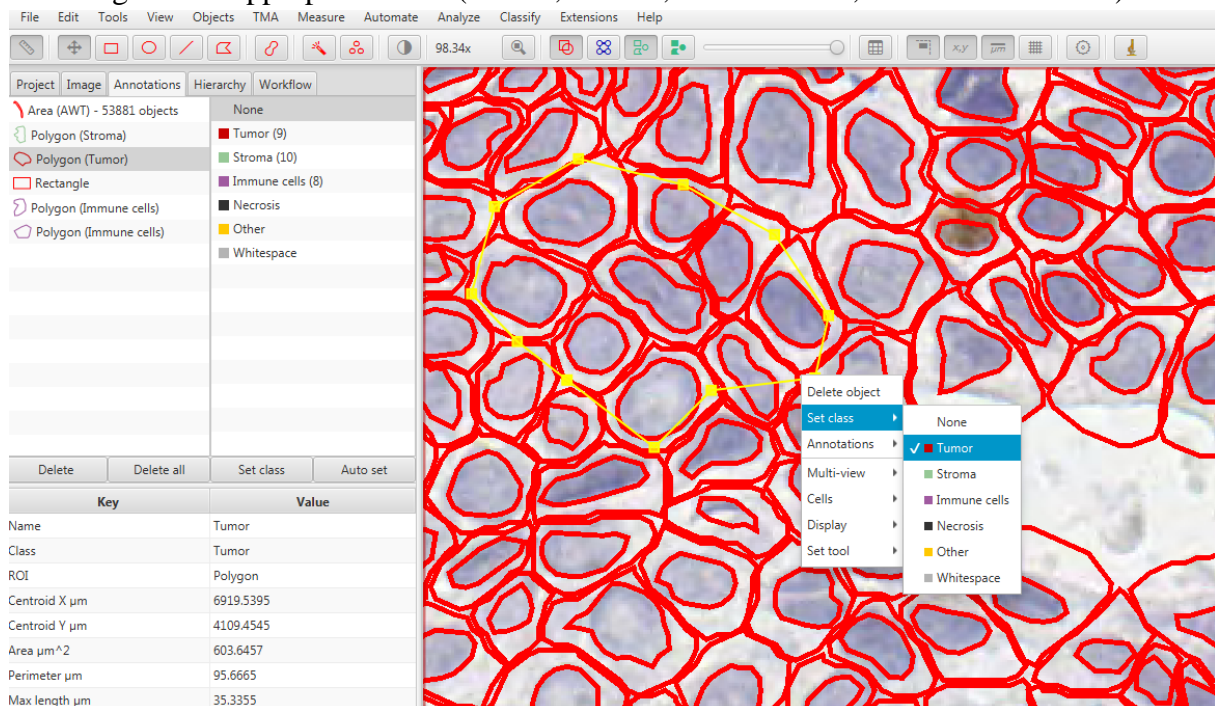
81 To set up a classifier for whole slides (WSI) use the slide 4 MIB1.

82 The next step is to begin annotating regions according to classes as follows: Tumor cells,
83 Stroma cells, Immune cells, Necrosis, Other. To annotate regions for classification, use the



84 polygon tool. It does not matter whether the detected cells are shown or hidden on the
85 image at the time; however, it can be helpful to toggle the detections on and off with the 
86 Show/hide detection objects tool while annotating. Try to annotate regions where the cells are
87 detected correctly (shape, size, cell count etc.). Use smaller annotations across the whole slide
88 to avoid the inclusion of different cell types and to represent more areas of the slide. Try to
89 annotate as many regions to represent the whole pattern of the slide and do not over- or
90 underrepresent any areas of the slide.

91 After an annotation has been drawn, right-click within the drawn annotation, then *Set class*
92 and assign to the appropriate class (Tumor, Stroma, Immune cells, Necrosis and Other).

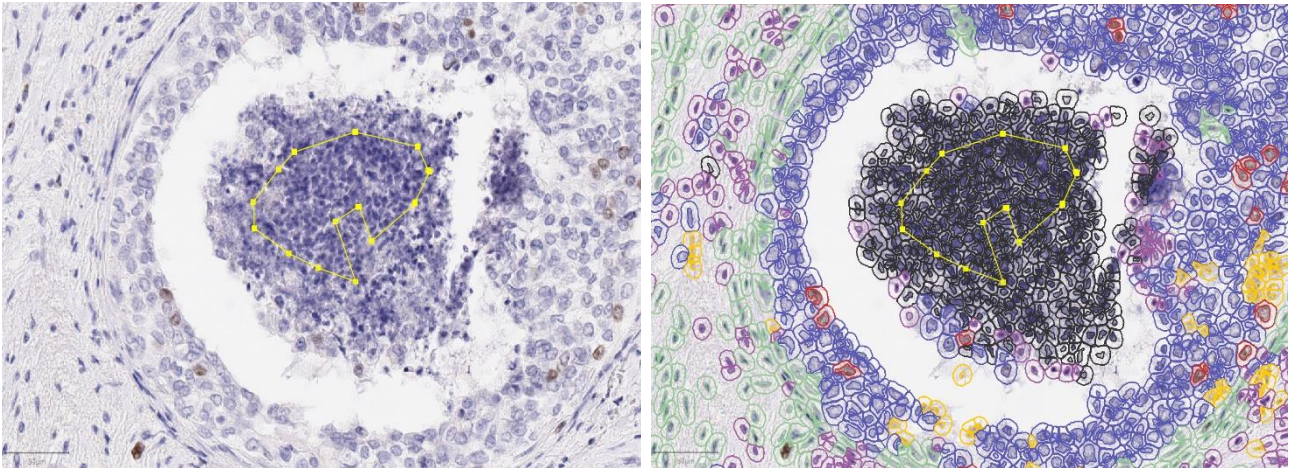


Key	Value
Name	Tumor
Class	Tumor
ROI	Polygon
Centroid X μm	6919.5395
Centroid Y μm	4109.4545
Area μm^2	603.6457
Perimeter μm	95.6665
Max length μm	35.3355

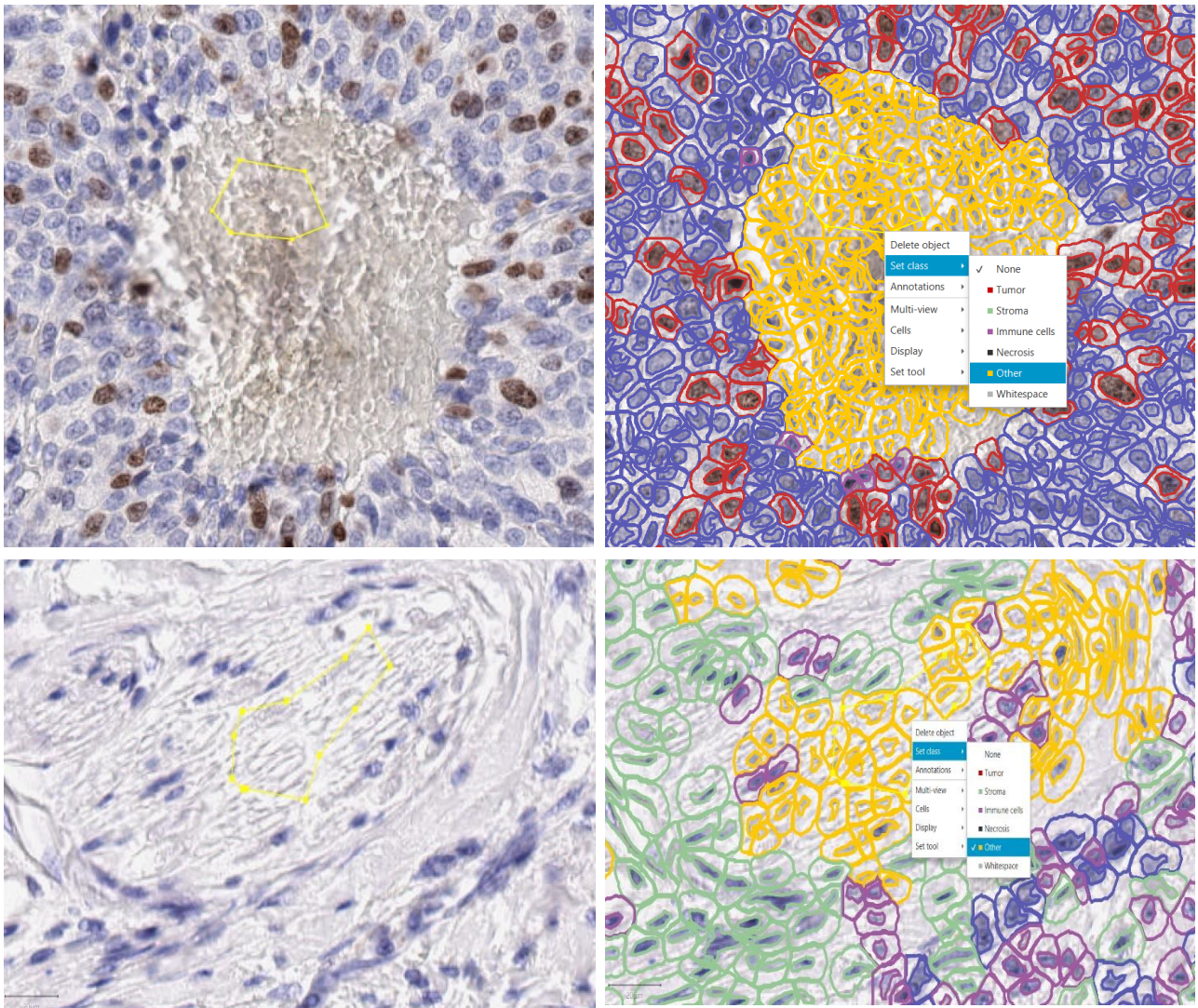
93
94 You should see the number increase beside the class that you selected (top right). This is the
95 number of cells inside all the annotations that you have drawn and assigned to this class.

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96 Annotate examples of necrosis (see images below) and assign them to *Necrosis* class.



97 Annotate examples of false cell detections (see images below) and a sign them to *Other* class.

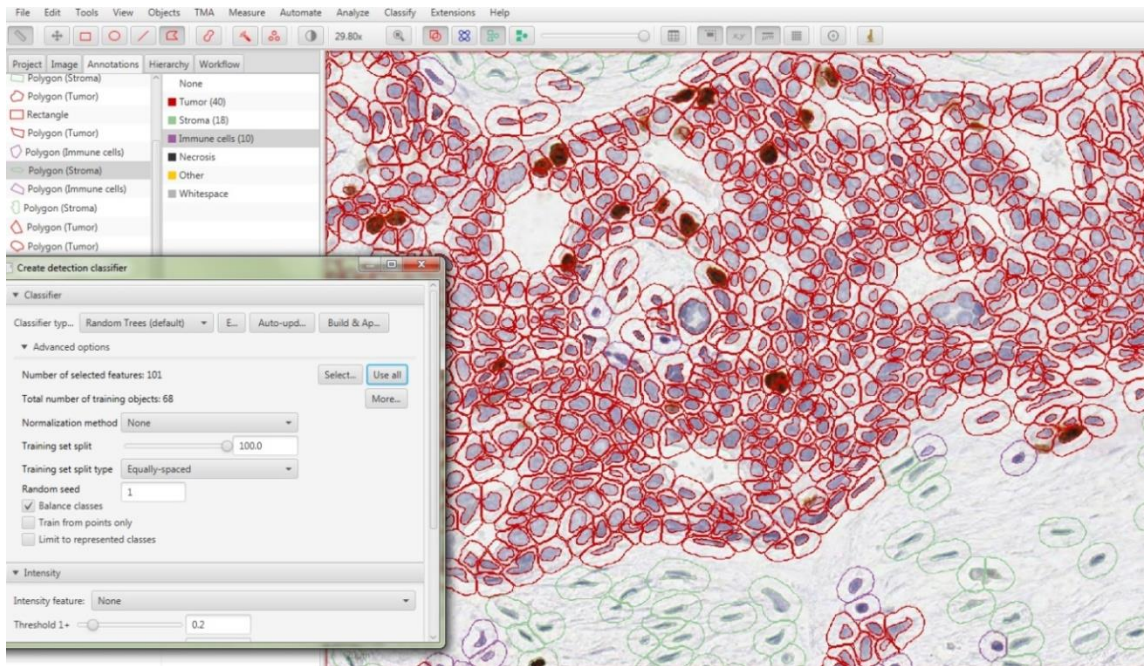


98 False cell detection can arise from stronger background, blood.

99 If you delete an annotation, make sure you keep the objects (click yes). Unless you want to
100 exclude cells or regions from the analysis. You should exclude large number of normal
101 epithelium, DCIS and artifacts (e.g.: irrelevant DAB staining) within invasive tumor areas that

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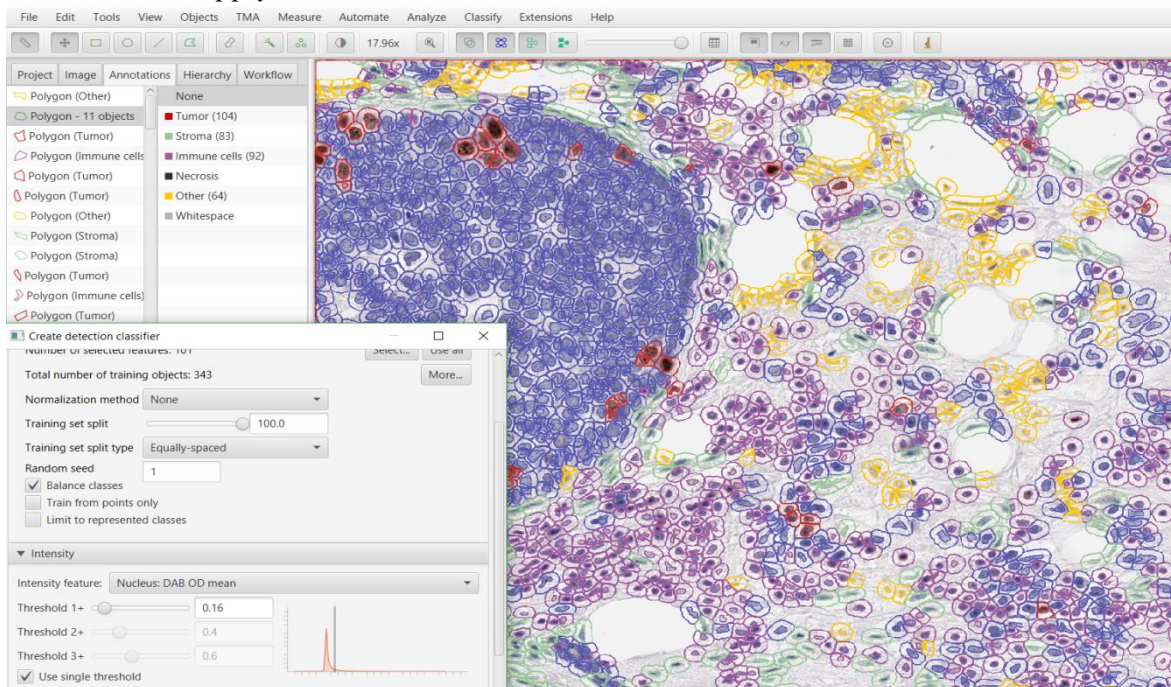
102 may influence the Ki67 LI score. To do this, annotate these areas, then delete those
103 annotations and do not keep the objects (click no). Small areas of normal tissue, DCIS do not
104 have significant impact on Ki67 LI, when you measure in this tumor cell count range.
105 Once you have several annotations with different classes, it is time to create the classifier to
106 see how well QuPath can distinguish the cells. Go to *Classify/Create detection classifier*.
107 Click on *Advanced options*, then click *Use all* button and select *Balance classes*. Pressing
108 *Build & Apply* will train up a classifier that QuPath will then apply to all cells within the
109 image (The color of the detected cells will correspond to color of its class).



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111 You can interactively improve the classification performance by adding annotations and set
112 their classes in areas that QuPath gets 'wrong', until you are satisfied with the performance.
113 After each annotation and setting its class, click *Build & Apply* to train the classifier.
114 Carefully review the whole slide searching for major misclassifications. The color of the
115 detected cells corresponds its class. The recommended minimum number of cell count ranges
116 from 150 -300 annotated cells for Tumor, Stroma and Immune cells classes.
117 Once you are satisfied with QuPath's performance to identify tumor cells, it is now time to
118 apply DAB staining intensity classification. For the *Intensity feature*, select *Nucleus: DAB*
119 *OD mean*. Set intensity threshold 1+ at 0.16 and check the box at *Use single threshold*. Then
120 click *Build & Apply*.



121
122 To apply the classifier again to the other slides, save this by clicking the *Save classifier* button
123 at the bottom of the classifier window. Also save the training objects by clicking *More/Save*
124 *training objects* in the classifier window. When you trained and saved the classifier and open
125 a new image, you have the option to *Retain current training objects in classifier*, select *Yes*.
126 When you open the next slide, you should run the estimate stain vectors, cell detection,
127 feature calculations as before, and then apply your pre-trained classifier as follows: From the
128 *Classify* menu select *Load classifier*. Then click *Load classifier* and select the classifier file
129 that you saved. The classifier will be immediately applied on the opened image. The loading
130 time depends on the image size.

131 **Step 5: View and export results**

132 If you select the original, large annotation containing all the cells then Ki67 labeling index
133 will appear in the lower measurements section of the *Annotations* tab on the left of the screen
134 as *Tumor: Positive %*. You can also generate results tables by clicking *Measure/Show*
135 *annotation measurements*. Click *Copy to clipboard* and past to an excel file.

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136 After you applied your core-biopsy trained classifier on the 30 core-biopsy slides (export
137 results into *CB-classifer_on_CB_slides* excel sheet), please apply it on the 30 whole slides
138 and export the results into a separate excel sheet (*CB-classifer_on_WSI* excel sheet). After
139 you applied your whole slide trained classifier on the 30 whole slides (export results into *WSI-*
140 *classifer_on_WSI* excel sheet), please apply it on the 30 core-biopsy slides and export the
141 results into a separate excel sheet (*WSI-classifer_on_CB_slides* excel sheet). Please be noted
142 that the classifiers are saved in the correspondent project (CB-classifier in CB project, WSI-
143 classifier in WSI project). Finally, make sure that your excel file (see the sample excel file)
144 contains only data of the large annotation for each slide. Name your excel file as:
145 *YourLabName_IKBCWG_Date*. Send your results, your 2 classifiers and your 2 training
146 objects files to Balazs Acs (balazs.acs@ki.se).

147 **If you have any questions, please contact Balazs Acs (balazs.acs@ki.se).**

148 **Reference:**

149 **All the information regarding how to use QuPath in more details can be found on**
150 **[QuPath documentation website](#).**

151 **If you are interested in how to batch process larger numbers of images in a reproducible**
152 **way, review the [Automation](#) section in QuPath documentation or contact Balazs Acs**
153 **(balazs.acs@ki.se). Please be noted, that the scripts should be used with caution and**
154 **attention: You should only use automation, if you understand each line in the script**
155 **(groovy language).**