

Supplementary data: Script file for semi-automated image analyses of HLA class I tumour epithelium expression in a tissue microarray (TMA) of rectal cancer using AxioVision digital image processing software (release 4.9.1, Zeiss).

General remarks: 1. This script file was written to analyse a batch of JPEG images for HLA class I tumour epithelium expression. In our study, we analysed all cores within a TMA section as one batch. 2. Steps 36 and 37 can also be performed using another program (e.g. Excel). 3. The .csv format was chosen to make the data interoperable. 4. All images produced during the analysis can be saved if wanted. 5. The script file contains words in Dutch. Translations can be found below.

Translations: Weefsel: Tissue; Epitheel: Epithelium; Resultaten: Results; Ruwe Data: Raw Data.

Command number	Check Box	Command type	Commands	Parameters	Comments	Explanations
1	y	Automatic	Run Macro	Macro = wait		The macro "wait" makes the computer wait for 100 ms. This time is used to empty working-memory.
2	y	Automatic	Run Macro	Macro = wait		The macro "wait" makes the computer wait for 100 ms. This time is used to empty working-memory.
3	y	Automatic	Run Macro	Macro = wait		The macro "wait" makes the computer wait for 100 ms. This time is used to empty working-memory.
4	y	Automatic	Run Macro	Macro = wait		The macro "wait" makes the computer wait for 100 ms. This time is used to empty working-memory.
5	y	Automatic	Run Macro	Macro = wait		The macro "wait" makes the computer wait for 100 ms. This time is used to empty working-memory.
6	n	Automatic	Close all	Force close = Yes		Closes all active windows containing tables and pictures.
7	y	Interactive	Specify filename (1)	Prefix = resultaten; uncheck all other options	User enters name for table of results	
8	y	Interactive	Comments	In next step select area to be analysed	Text in Column E is shown to user	Information for user.
9	y	Automatic	Binary Image Editor	Mask Image = ActiveImage Densimage = ActiveImage Output = EditedImage Mode = Interactive Reset = Yes Binary Image Editor mode = AddObjects Settings = Ellipse Propagate mask = empty		Manual pre-selection of area of interest. Allows user to remove unwanted areas from analysis (Necrosis, small tissue fragments etc.).
10	y	Automatic	Threshold RGB	Input = OpenImage Output = Weefsel LevelLowRed = 0 LevelHighRed = 255 LevelLowGreen = 0 LevelHighGreen = 218 LevelLowBlue = 0 LevelHighBlue = 255 Create binary image = No	Values for RGB were set once for every staining. Level value 0 = dark, and level value 255 = bright. A pixel with values 0,0,0 for R,G,B is black.	Selection of tissue within area of interest. Areas containing no tissue are excluded. Settings should be determined for every series of sections stained.

				Invert result = No		
11	y	Automatic	And	Input1 = Weefsel Input2 = EditedImage Output = Weefsel2		Selection of pixels selected in both "Weefsel" and "EditedImage".
12	y	Automatic	Dilate	Input = Weefsel2 Output = Weefsel3 Structuring Element = Cross Count = 3	Values are specific for the image resolution used	Fine-tuning of tissue selection. The result is a binary image of the selection.
13	y	Automatic	Erode	Input = Weefsel3 Output = Weefsel4 Structuring Element = Octagon Count = 3	Values are specific for the image resolution used	Fine-tuning of tissue selection. The result is a binary image of the selection.
14	y	Automatic	And	Input1 = Active Image Input2 = Weefsel4 Output = Weefsel5		Selection of pixels selected in both "Active Image" and "Weefsel4" in which "Active Image" is the original image. The result is a color image of the tissue named "Weefsel5".
15	y	Automatic	Load Measurement Properties	Measurement properties file = AreaPixel1.zma	Defined below	Loading file containing "instructions" for the measurement.
16	y	Automatic	Start Measurement	DensImage = ActiveImage MaskImage = Weefsel5 DrawImage = DrawWeefsel Region-specific table = RegionWeefsel Field-specific table = FieldWeefsel Mode = Index Draw in = DrawImage		Performs measurement as dictated by the Measurement Properties. Results of the measurement(s) are shown in a region-specific table, a field-specific table and an image (DrawWeefsel) in which the mask image in annotated in the original image. Results include the area (in pixels squared) of the tissue.
17	y	Interactive	Comments	In the next step select tumor epithelium	Text in Column E is shown to user	Information for user.
18	y	Automatic	Thresholds interactive	Input = Weefsel5 Output = Epitheel1 Level Low and Level High for Hue, Luminance and Saturation Create binary image = Yes Invert result = No Remove unsegmented = No Mode = Interactive ColorModel = HLS	Levels Low and High for Hue, Luminance and Saturation are set for every core during analysis to prevent selection of background staining as tumor epithelium. Level value 0 = dark, and level value 255 = bright. A pixel with values 0,0,0 for R,G,B is black.	Pre-selection of tumor epithelium.
19	y	Automatic	Scrap	Input = Epitheel1 Output = Epitheel2 MinArea = 1 MaxArea = 500 Select = No	MaxArea is specific for the image resolution used	Removal of areas too small to be tumor epithelium.
20	y	Automatic	Dilate	Input = Epitheel2 Output = Epitheel3 Structuring Element = Cross Count = 5	Values are specific for the image resolution used	Fine-tuning of tissue selection. The result is a binary image of the selection.

21	y	Automatic	Erode	Input = Epiteel3 Output = Epiteel4 Structuring Element = Octagon Count = 3	Values are specific for the image resolution used	Fine-tuning of tissue selection. The result is a binary image of the selection.
22	y	Automatic	And	Input1 = Weefsel5 Input2 = Epiteel4 Output = Epiteel5		Selection of pixels selected in both "Weefsel5" and "Epiteel4". The result is a color image of the tumor epithelium named "Epiteel5".
23	y	Automatic	Load Measurement Properties	Measurement properties file = AreaPixel1.zma	Defined below	Loading file containing "instructions" for the measurement.
24	y	Automatic	Start Measurement	DensImage = ActiveImage MaskImage = Epiteel5 DrawImage = DrawEpiteel Region-specific table = RegionEpiteel Field-specific table = FieldEpiteel Mode = Index Draw in = DrawImage		Performs measurement as dictated by the Measurement Properties. Results of the measurement(s) are shown in a region-specific table, a field-specific table and an image ("DrawEpiteel") in which the mask image is annotated in the original image. The results include the area (in pixels squared) of the tumor epithelium.
25	y	Automatic	Convert Pixel Format	Input = Epiteel5 Output = ConvertPixelFormatImage PixelFormat = 8bit Keep Range = Off		For some stains it is known that the color-composition (as in RGB) alters with the intensity. To prevent any problems with the selection of positively stained tumor epithelium we convert the image to 8bit black and white.
26	y	Interactive	Comments	In the next step select positive tumor epithelium	Text in Column E is shown to user	Information for user.
27	y	Automatic	Threshold Interactive	Input = ConvertPixelFormatImage Output = EpiteelPos1 LevelLowRed = 1 LevelHighRed = 195 LevelLowGreen = 1 LevelHighGreen = 195 LevelLowBlue = 1 LevelHighBlue = 195 Create binary image = Yes Invert result = no Remove unsegmented = No Automatic = No Mode = Interactive ColorModel = RGB	Settings for levels are identical for all cores. Levels for RGB are identical because the picture is in grey-scale. The software has no threshold-option in grey-scale. Level value 0 = dark, and level value 255 = bright. A pixel with values 0,0,0 for R,G,B is black.	Pre-selection of positively stained tumor epithelium.
28	y	Automatic	Scrap	Input = EpiteelPos1 Output = EpiteelPos2 MinArea = 1 MaxArea = 100 Select = No	MaxArea is specific for the image resolution used	Removal of areas too small to be positively stained tumor epithelium.
29	y	Automatic	Dilate	Input = EpiteelPos2 Output = EpiteelPos3 Structuring Element = Octagon Count = 7	Values are specific for the image resolution used	Fine-tuning of tissue selection. The result is a binary image of the selection.

30	y	Automatic	Erode	Input = EpitheelPos3 Output = EpitheelPos4 Structuring Element = Octagon Count = 6	Values are specific for the image resolution used	Fine-tuning of tissue selection. The result is a binary image of the selection.
31	y	Automatic	And	Input1 = EpitheelPos4 Input2 = Epitheel5 Output = EpitheelPos5		Selection of pixels selected in both "EpitheelPos4" and "Epitheel5". The result is a color image of the positively stained tumor epithelium named "EpitheelPos5".
32	y	Automatic	Load Measurement Properties	Measurement properties file = DensMean.zma	Defined below	Loading file containing "instructions" for the measurement.
33	y	Automatic	Start Measurement	DensImage = ActiveImage MaskImage = EpitheelPos5 DrawImage = DrawEpitheelPos Region-specific table = RegionEpitheelPos Field-specific table = FieldEpitheelPos Mode = Index Draw in = DrawImage		Performs measurement as dictated by the Measurement Properties. Results of the measurement(s) are shown in a region-specific table, a field-specific table and an image ("DrawEpitheelPos) in which the mask image in annotated in the original image. The results include the area (in pixels squared) of the positively stained tumor epithelium.
34	y	Interactive	Save Image As...	Document = DrawEpitheelPos Folder = "path" Use Source Folder = No Name = Name Type = zvi Name definition = Custom Force save = no Burn-in Annotations = Yes Apply display mapping = Yes Convert to 8bit = No Quality = 90	.zvi is a file-type, used by AxioVision, that contains data about the image (magnification, image size etc.) User gives a name to each picture manually.	Interactive step in which the user enters a name for the "DrawEpitheelPos" image for storage. Other filetypes can be selected (JPEG, TIFF etc.).
35	y	Automatic	Calculate table columns	Input table 1 = FieldEpitheel Input table 2 = FieldWeefsel Output table = Output Table 1 Column Selection 1 = 1 Factor 1 = 100 Offset 1 = 0 Column selection 2 = 1 Factor 2 = 1 Offset 2 = 0 Operation = Divide New column name = % TumorEpitheel	Column-order is defined in the Measurement properties	Calculates the percentage of tumor epithelium in the tissue.
36	y	Automatic	Calculate table columns	Input table 1 = FieldEpitheelPos Input table 2 = FieldEpitheel Output table = Output Table2 Column Selection 1 = 1 Factor 1 = 100 Offset 1 = 0 Column selection 2 = 1 Factor 2 = 1 Offset 2 = 0 Operation = Divide	Column-order is defined in the Measurement properties	Calculates the percentage of positively stained tumor epithelium within the tumor epithelium.

				New column name = % TumorEpitheelPos		
37	y	Automatic	Join table columns	Input table 1 = FieldWeefsel Input table 2 = Output Table1 Output table = Output Table3 Column selection 1 = 1,2 Column selection 2 = 1	Column-order is defined in the Measurement properties	Combines columns of two tables in one table.
38	y	Automatic	Join table columns	Input table 1 = Output Table3 Input table 2 = Output Table2 Output table = Resultaten Column selection 1 = 1,2,3 Column selection 2 = 1	Column-order is defined in the Measurement properties	Combines columns of two tables in one table.
39	y	Automatic	Append table	Input table = Resultaten Output table = Ruwe Data		Combines result of multiple cores in one file.
40	y	Automatic	Save Data table As...	Document = Ruwe Data Folder = "path" Use Source Folder = No Name = "name" Name definition = Specify filename (1) Force save = No Extension = csv	"name" is name as given in step 7	Autosaves the results in a file named in step 7.
41	y	Automatic	Close	Document name = Resultaten Force close = Yes		Closes the table containing the results.

Measurement properties:

Area Pixel1.zma	In both "Region Features" and "Field Features" the following "Meas Features" are chosen: ImageName and Area. In "Region Features" the "Draw Feature" "DrawContour" is chosen. For both "Region Features" and "Field Features" a table of results is created; a "Region-specific" table and a "Field-specific" table (see parameters in Measurement steps). The Region-specific table consists of two columns: "ImageName" stating the file name of the analysed image and "Area" stating the area (in pixels squared) for every selected region. The Field-specific table also consists of two columns: "ImageName" stating the file name of the analysed image and "Area" stating the total area (in pixels squared) of all regions selected. The "DrawImage" that is created shows the "DensImage" in which the regions selected are outlined.
DensMean.zma	In "Region Features" we chose the options "Area" and "Denistometricmean" in the "Meas Features" and "DrawContour" in the "Draw Features". In "Field Features" we chose the option "Areasum". The "Region-specific" table consists of two columns: "Area" stating the area (in pixels squared) of every region selected and "Densitometricmean" stating the densitometric mean of every region selected. The "Field-specific" table consist of one column: "Areasum" stating the total area (in pixels squared) of all regions selected. The "DrawImage" that is created shows the "DensImage" in which the regions selected are outlined. The results of the Densitometric mean were not used in this paper.