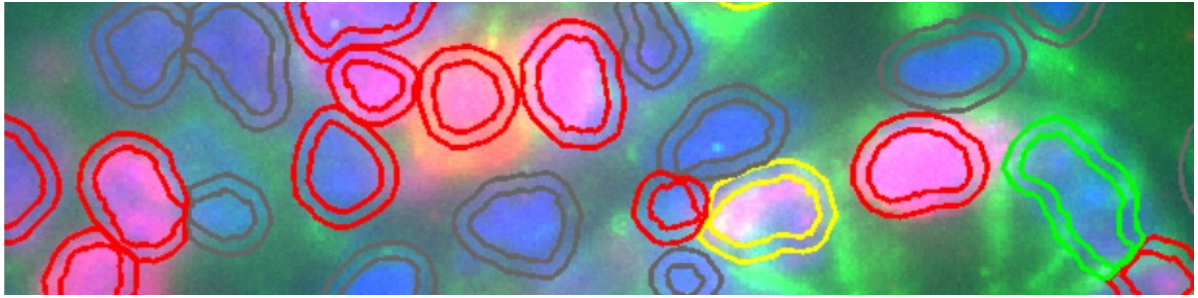
March 2026

Chapter 7

Multiplex Analysis



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Multiplex Analysis

Stained nuclei

- For cell detection

Different stains

- Count cells positive for each stain
- Count cells positive for any combination of stains

DAPI, C1, C2, C3

Number of cells positive for

- C1, C2, C3,
  C1C2, C2C3, C1C3, C1C2C3

Multiplex Analysis

Plan

- Set channel names and colors
- Detect all cells
- Train classifiers for each stain
- Create a combined classifier
- Apply the combined classifier to the project
- Reorganize the output data
  - Count each positive detection whether single or in combination

$$c1\_total = c(C1) + c(C1C2) + c(C1C3) + \ldots + c(C1C2C3)$$

Set channel names and colors

We will first set the channel names

They correspond to the markers of interest

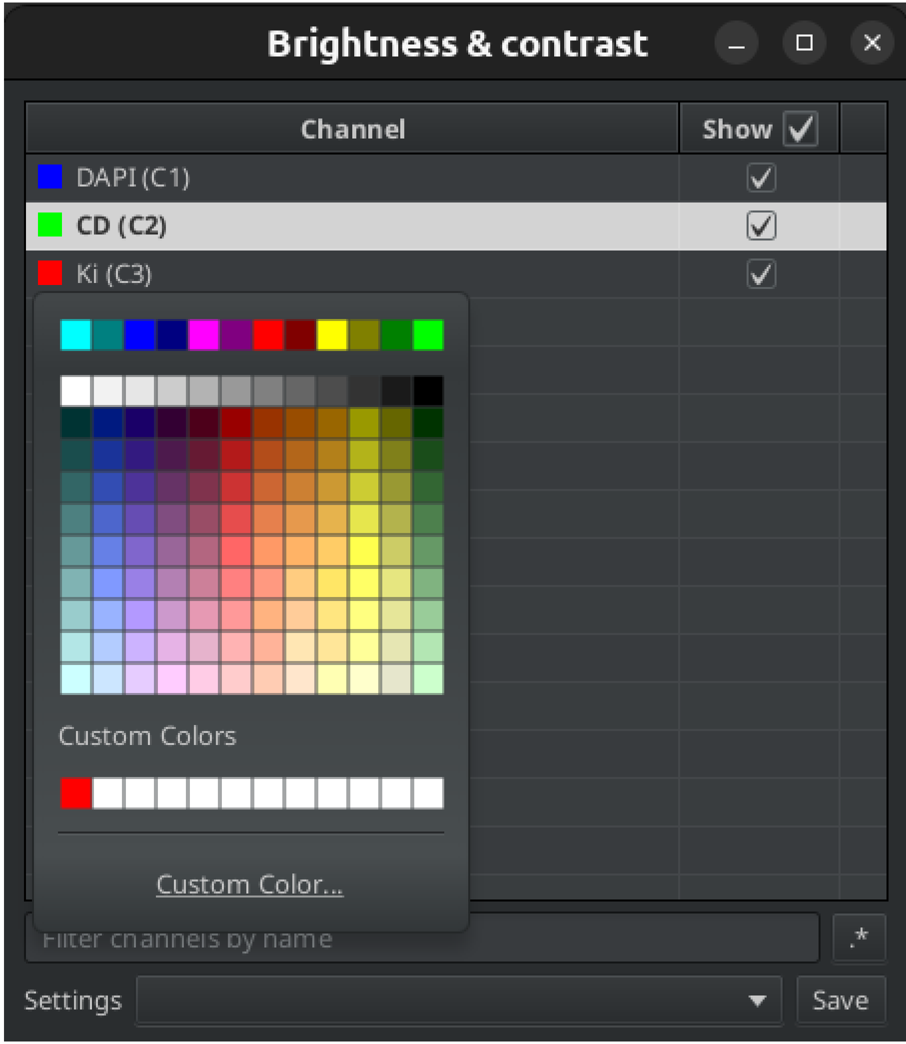
We will create classifiers from them

For one image, use B&C tool

Hoover over the color and note the hex-string of the color

For example

- #ff0000 (red),
- #00ff00 (green)
- #0000ff (blue)



Set channel names and colors

Use a script to apply to all images in the project

Run for project

0, 1, 2, … A, B, C, D, E, F
0, 10, 15

$D_2D_{1\ \text{base}\ 16} = D2 * 16 + D1 * 1$

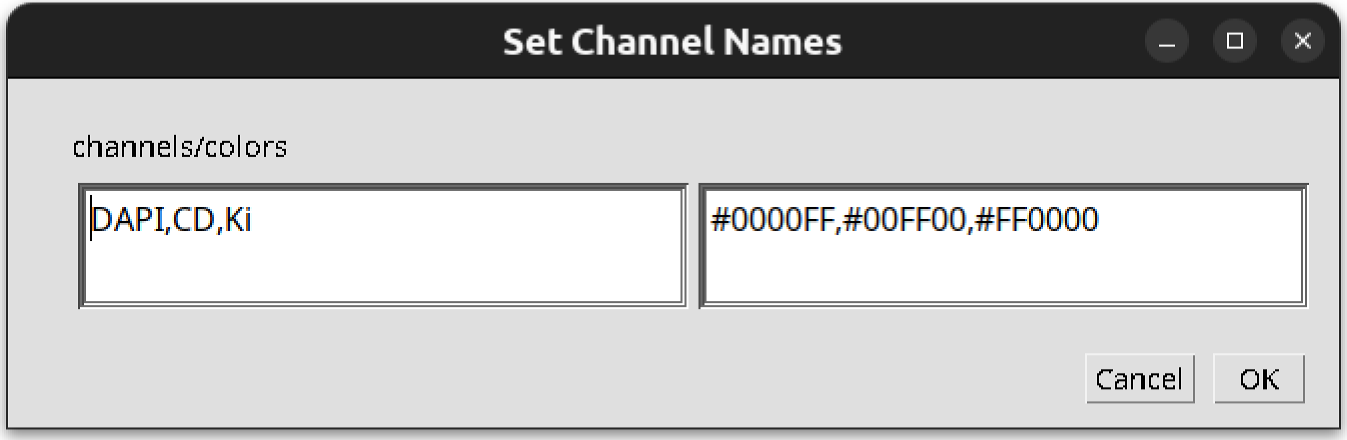
For example F5 = 15*16 + 5 = 245

```
setChannelNames("PDL1","CD8","FoxP3","CD68","PD1","CK","DAPI","AF")
setChannelColors(
    getColorRGB(255, 255, 255),
    getColorRGB(0, 255, 0)
)
setChannelDisplayRange("CD8",0, 5)
```

Set channel names and colors

Download and run (for project) set_channel_names.groovy from

https://github.com/MontpellierRessourcesImagerie/qupath_multiplex_analysis



DAPI,CD,Ki

#0000FF,#00FF00,#FF0000

Cell detection

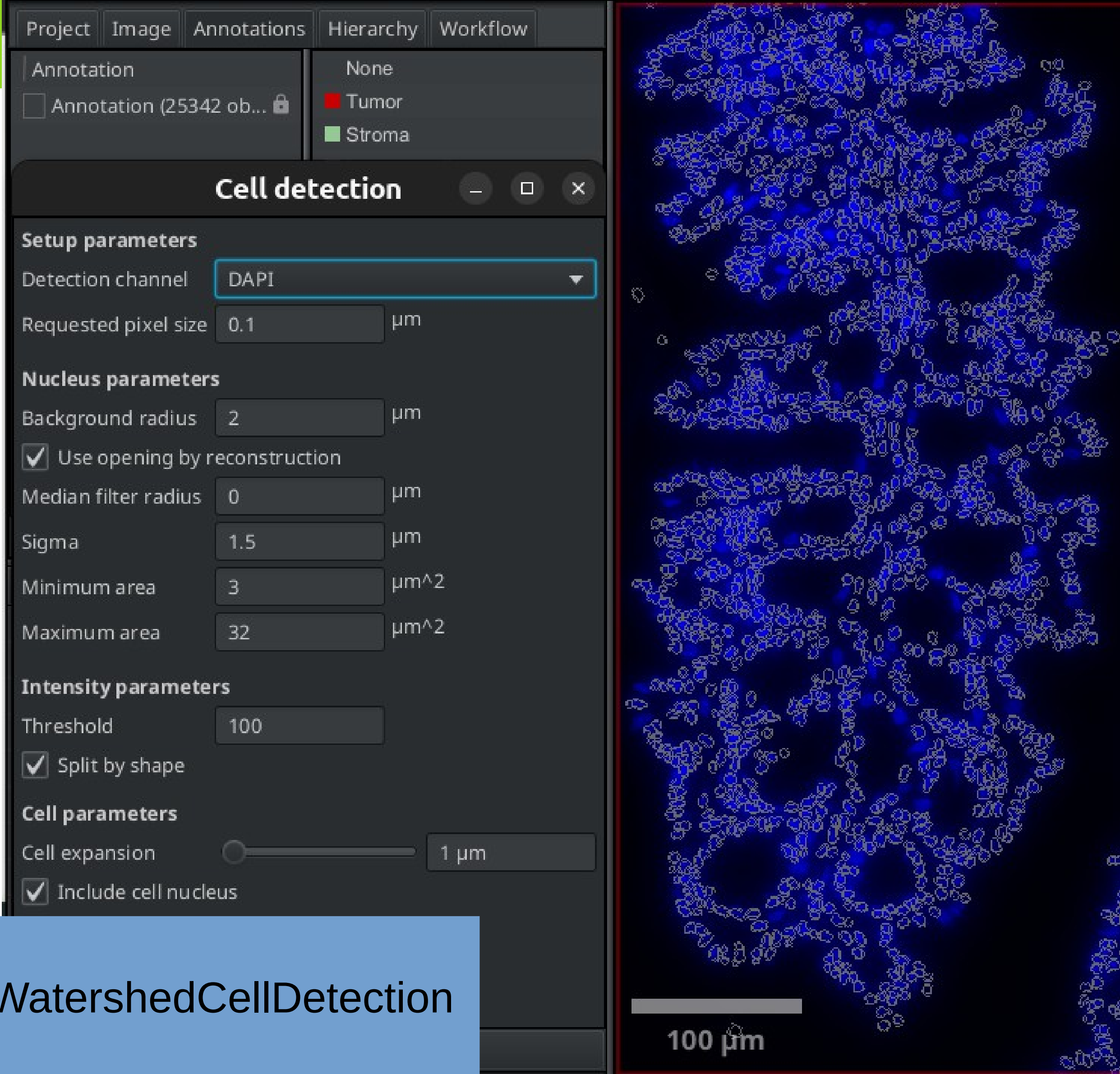
Analyze>
Cell Detection>
Cell Detection or

Cellpose

Stardist

Apply to whole project

- Create command history script
- Run for project



Cell detection

Setup parameters

Detection channel: DAPI
Requested pixel size: 0.1 µm

Nucleus parameters

Background radius: 2 µm
☑ Use opening by reconstruction
Median filter radius: 0 µm
Sigma: 1.5 µm
Minimum area: 3 µm^2
Maximum area: 32 µm^2

Intensity parameters

Threshold: 100
☑ Split by shape

Cell parameters

Cell expansion: 1 µm
☑ Include cell nucleus

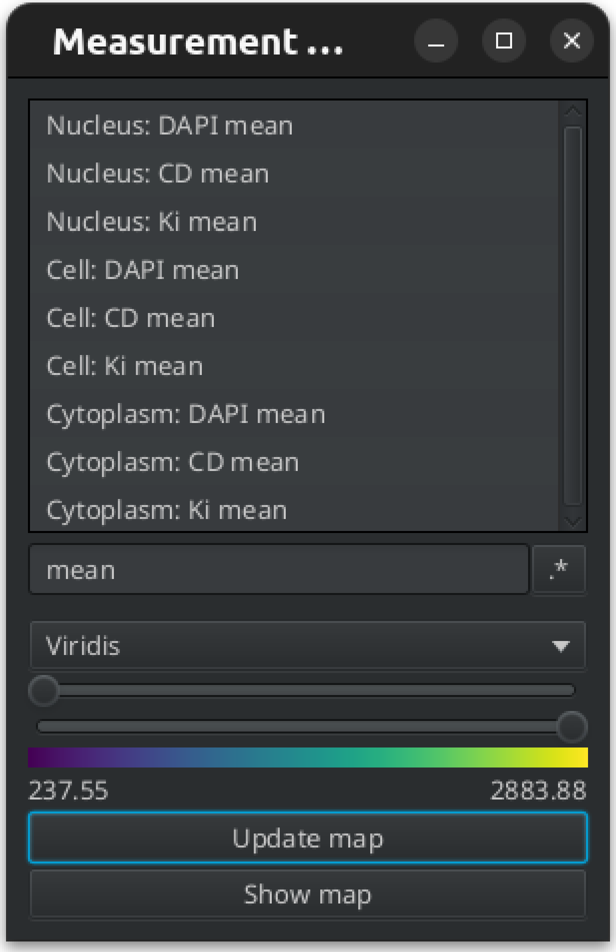
```
setImageType('FLUORESCENCE');
runPlugin('qupath.imagej.detect.cells.WatershedCellDetection
'
```

Cell Detection Measurements

Cell Detection

- Automatically measures features for
  - cell
  - cytoplasm
  - nucleus

We will train the classifiers with these features

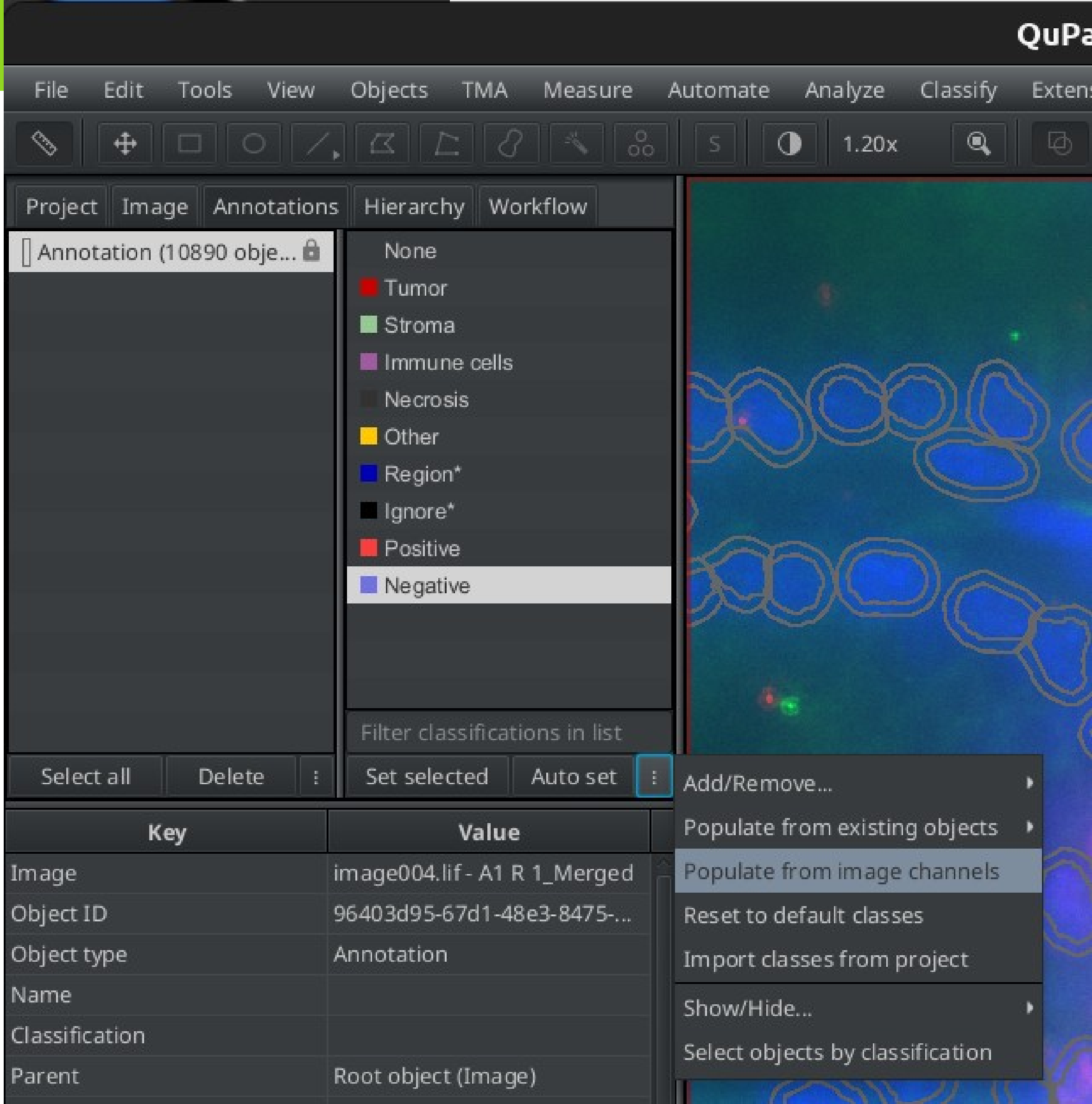


Nucleus: DAPI mean
Nucleus: CD mean
Nucleus: Ki mean
Cell: DAPI mean
Cell: CD mean
Cell: Ki mean
Cytoplasm: DAPI mean
Cytoplasm: CD mean
Cytoplasm: Ki mean

Train a classifier per channel

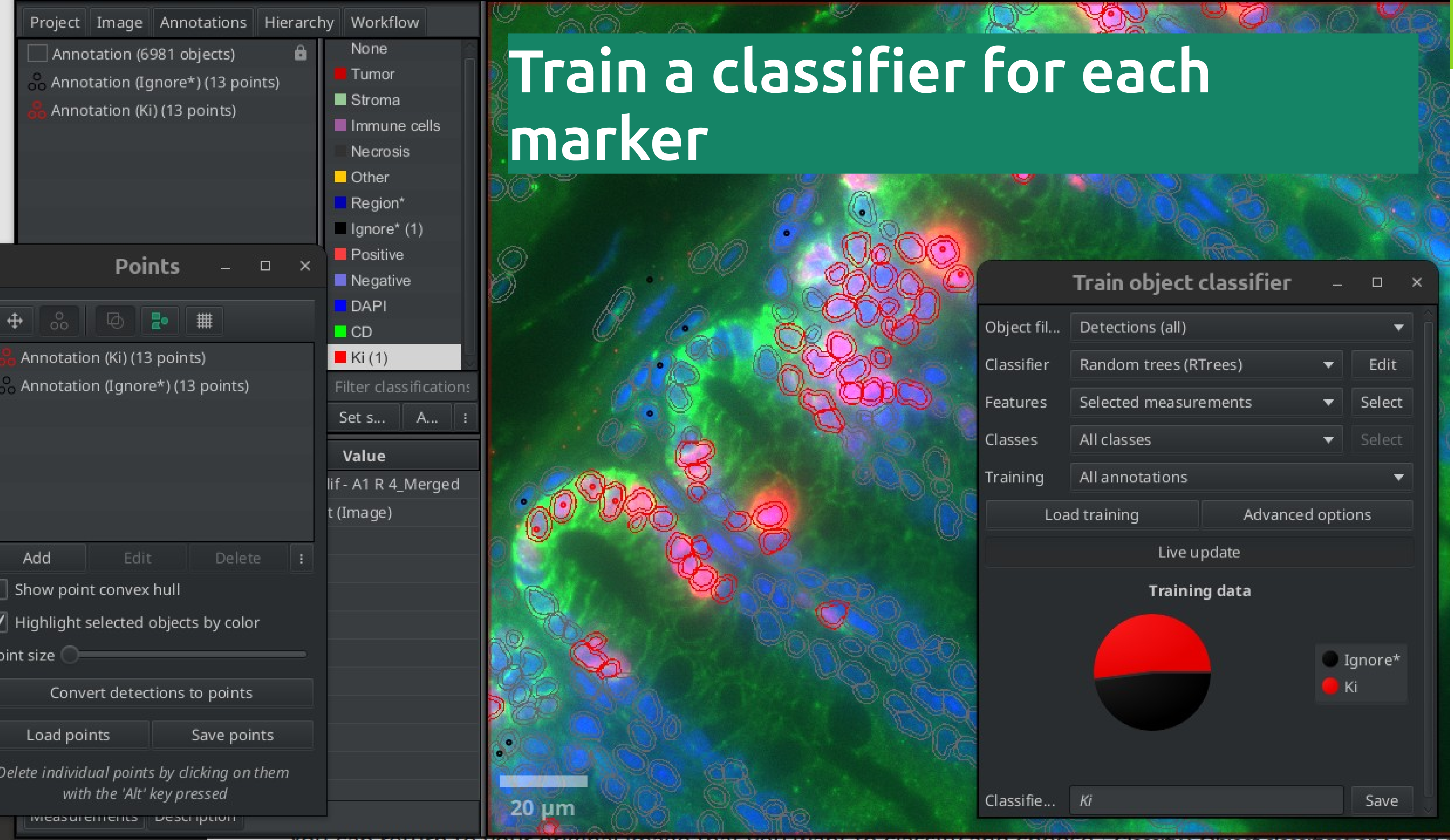
Populate from image channels

- Creates a class for each channel project not for an image
- No need to create a script and “run for project” in this case



Key	Value
Image	image004.lif - A1 R 1_Merged
Object ID	96403d95-67d1-48e3-8475-...
Object type	Annotation
Name	
Classification	
Parent	Root object (Image)

Train a classifier for each marker



Annotation (6981 objects)
Annotation (Ignore*) (13 points)
Annotation (Ki) (13 points)

Object fil...	Detections (all)
Classifier	Random trees (RTrees)
Features	Selected measurements
Classes	All classes
Training	All annotations
Classifie...	Ki

Training data

Ignore*
Ki

lif - A1 R 4_Merged
t (Image)

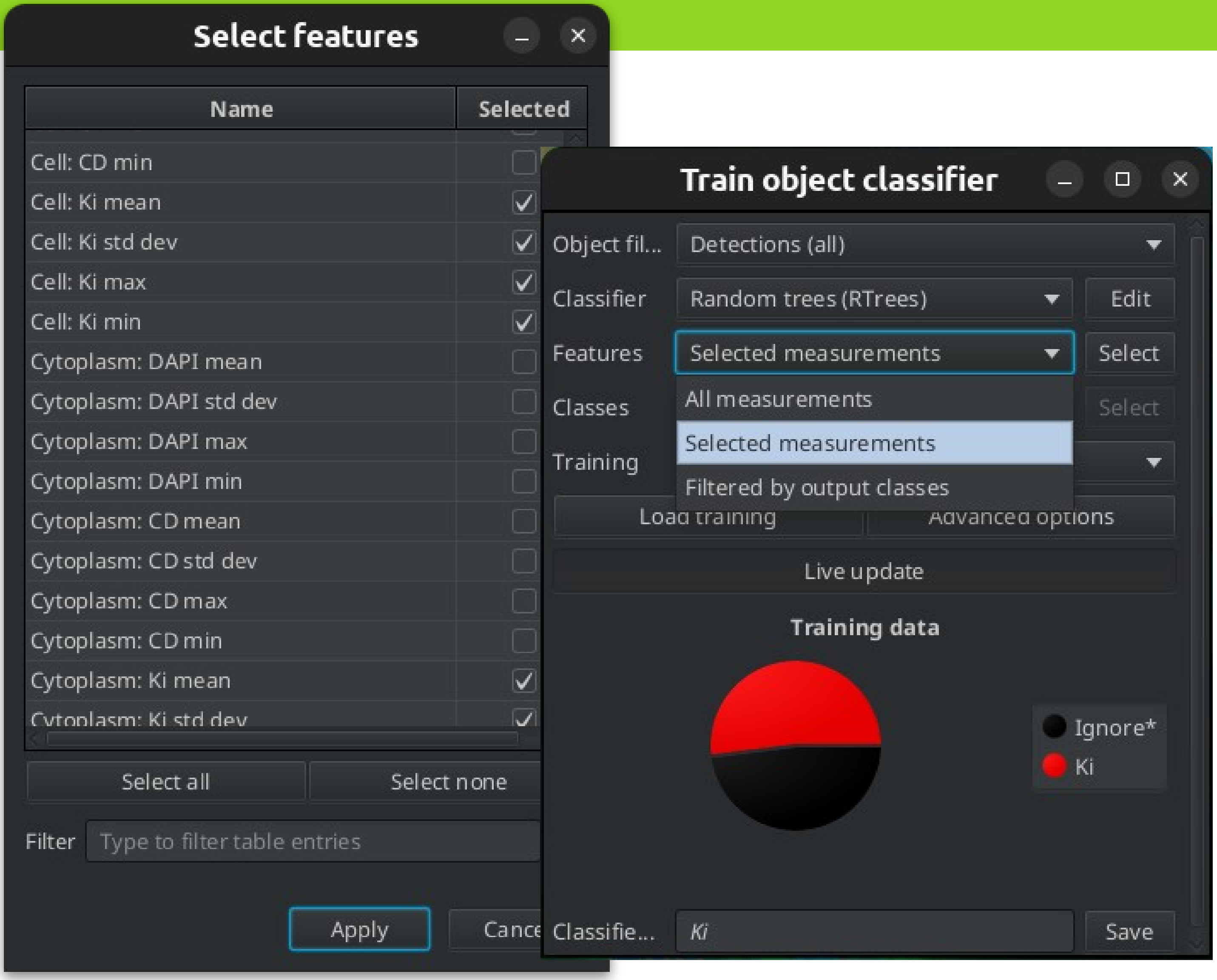
Train a classifier for a marker

Select measurements

- Select mostly measurements of the marker

Filtered by output class

- Selects all measurements containing the class name



Select features

Name	Selected
Cell: CD min	☐
Cell: Ki mean	☑
Cell: Ki std dev	☑
Cell: Ki max	☑
Cell: Ki min	☑
Cytoplasm: DAPI mean	☐
Cytoplasm: DAPI std dev	☐
Cytoplasm: DAPI max	☐
Cytoplasm: DAPI min	☐
Cytoplasm: CD mean	☐
Cytoplasm: CD std dev	☐
Cytoplasm: CD max	☐
Cytoplasm: CD min	☐
Cytoplasm: Ki mean	☑
Cytoplasm: Ki std dev	☑

Train object classifier

Object fil...: Detections (all)

Classifier: Random trees (RTrees)

Features: Selected measurements

Classes

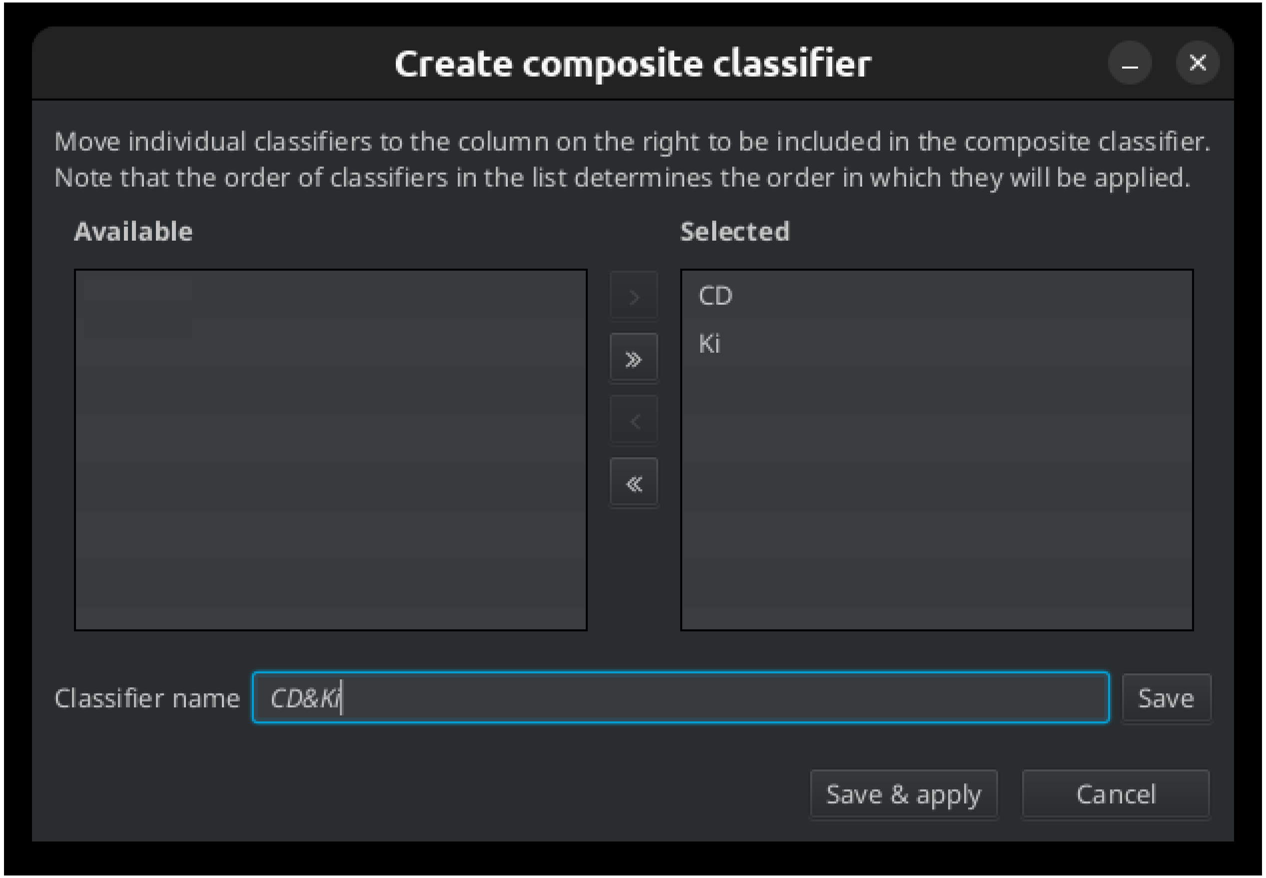
Training

Classifie...: Ki

Composite Classifier

Create and save a composite classifier

Classify>
Object Classificatrion>
Create composite classifier



Move individual classifiers to the column on the right to be included in the composite classifier. Note that the order of classifiers in the list determines the order in which they will be applied.

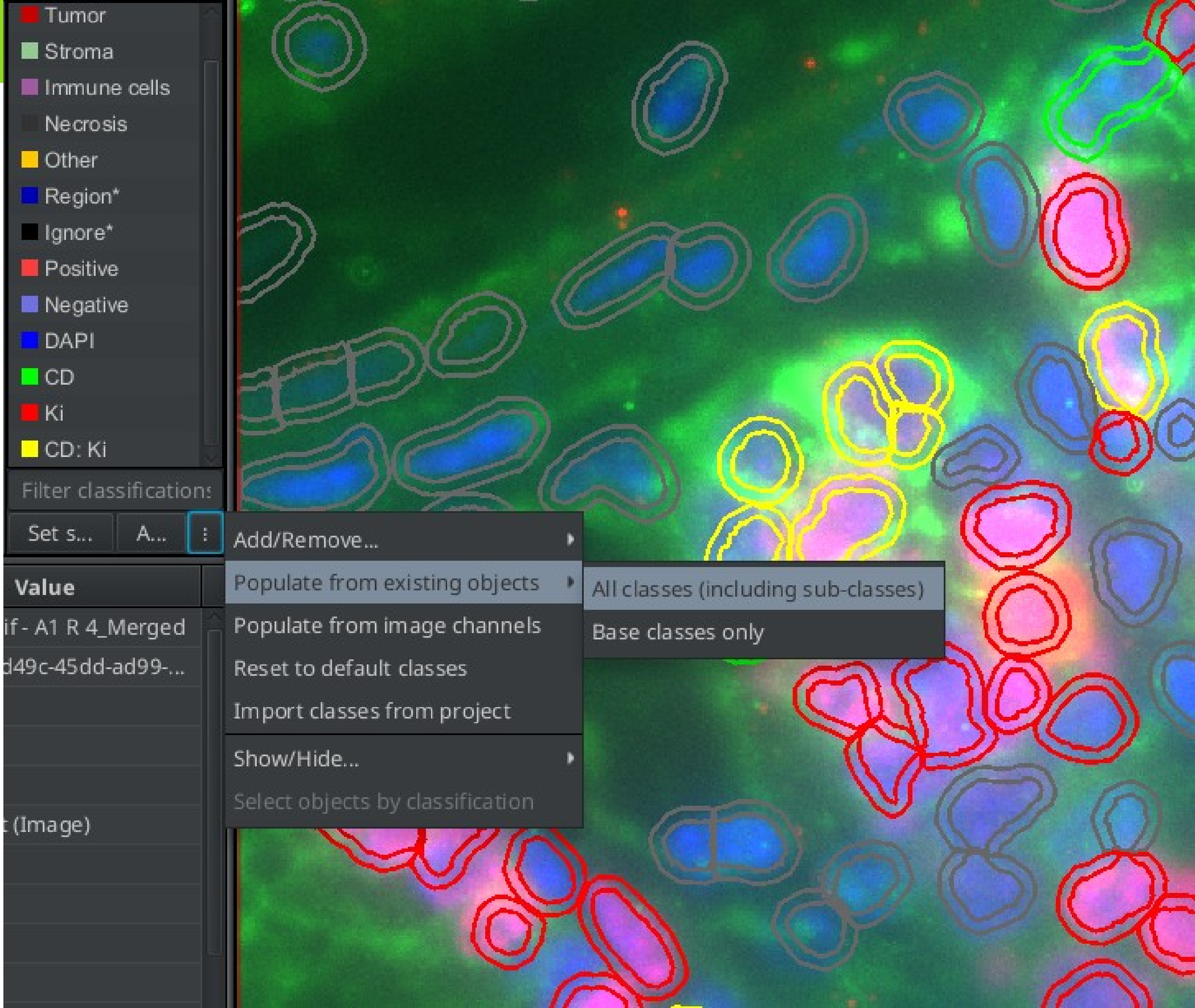
Selected: CD, Ki

Classifier name: CD&Ki

View classifications

Populate from existing objects>all classes

- Will add the combined class(es)
- You can change colors
    - To distinguish them from ignored cells

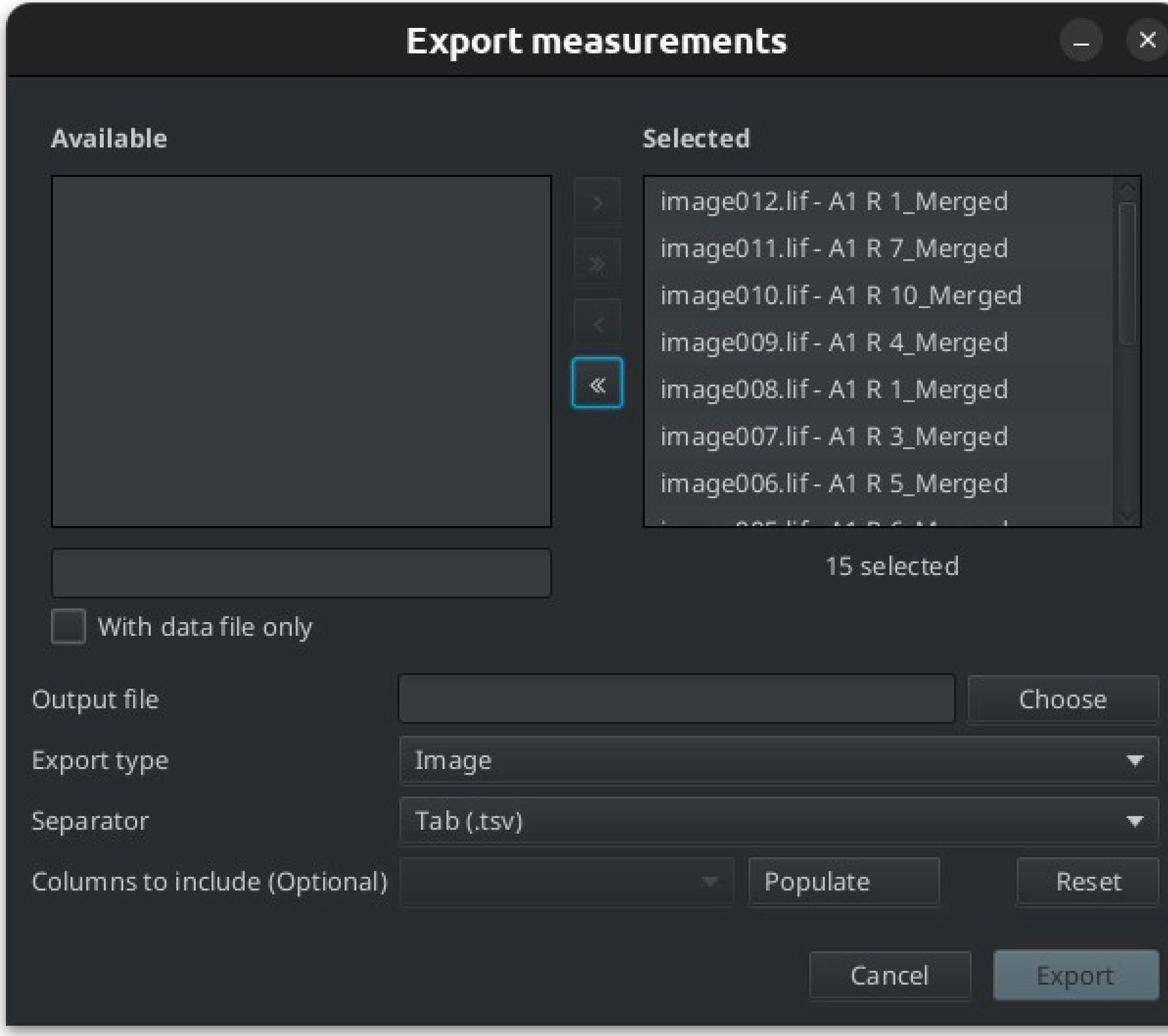


Run on project

Automate>Create command history script

run for project

```
runObjectClassifier("CD&Ki");
```



Export measurements

Available

Selected

image012.lif - A1 R 1_Merged
image011.lif - A1 R 7_Merged
image010.lif - A1 R 10_Merged
image009.lif - A1 R 4_Merged
image008.lif - A1 R 1_Merged
image007.lif - A1 R 3_Merged
image006.lif - A1 R 5_Merged

15 selected

☐ With data file only

Output file:

Export type: Image

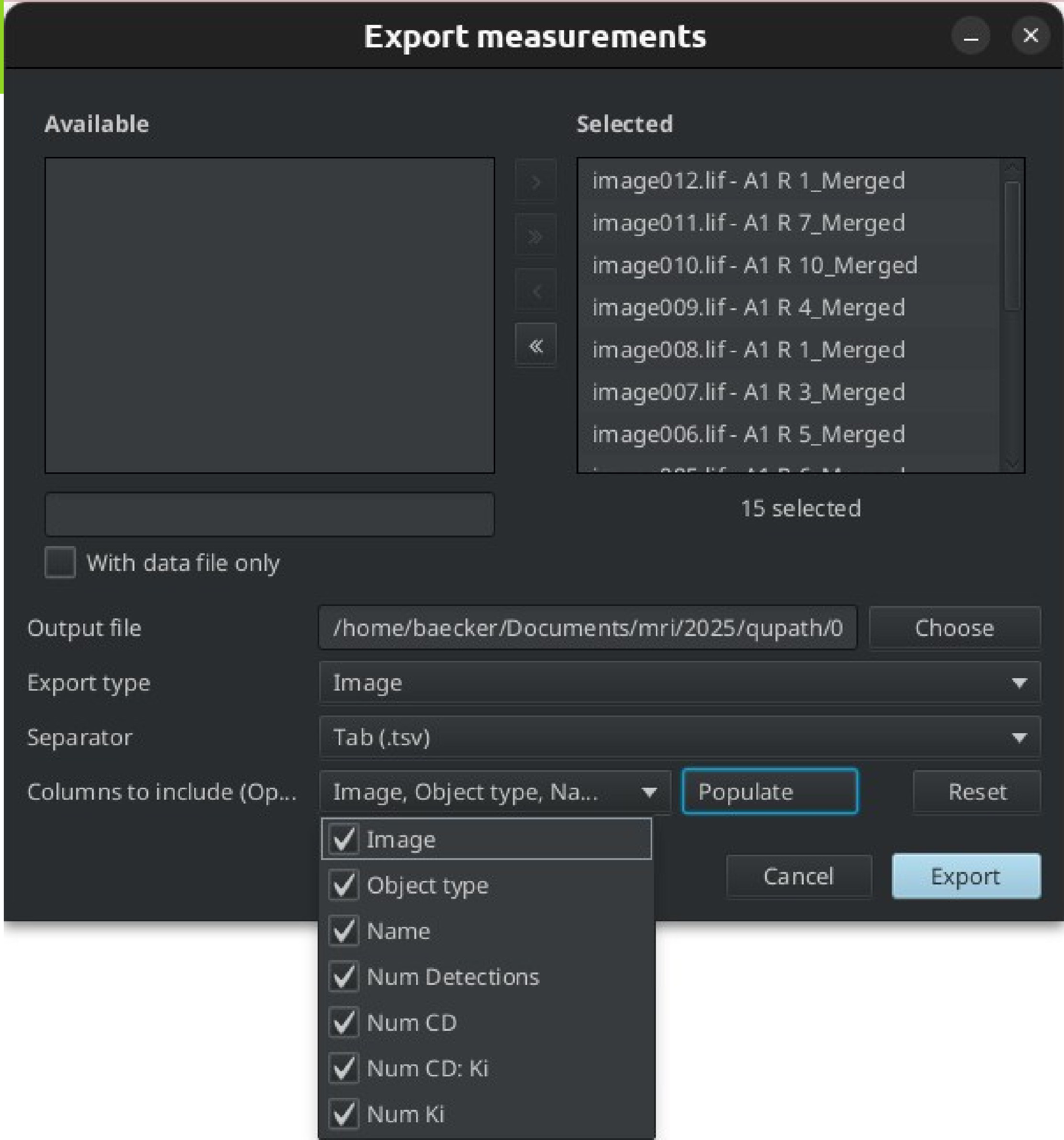
Separator: Tab (.tsv)

Columns to include (Optional):

Export Measurements

Measure>Export Measurements

Export Image or Annotations or Detections



Export measurements

Available

Selected

image012.lif - A1 R 1_Merged
image011.lif - A1 R 7_Merged
image010.lif - A1 R 10_Merged
image009.lif - A1 R 4_Merged
image008.lif - A1 R 1_Merged
image007.lif - A1 R 3_Merged
image006.lif - A1 R 5_Merged

15 selected

☐ With data file only

Output file: /home/baecker/Documents/mri/2025/qupath/0

Export type: Image

Separator: Tab (.tsv)

Columns to include (Op...: Image, Object type, Na...

☑ Image
☑ Object type
☑ Name
☑ Num Detections
☑ Num CD
☑ Num CD: Ki
☑ Num Ki

Measurements

Problem

- Num CD counts CD positive cells that are not Ki positive

- Add sum of columns in excel or
- run the script

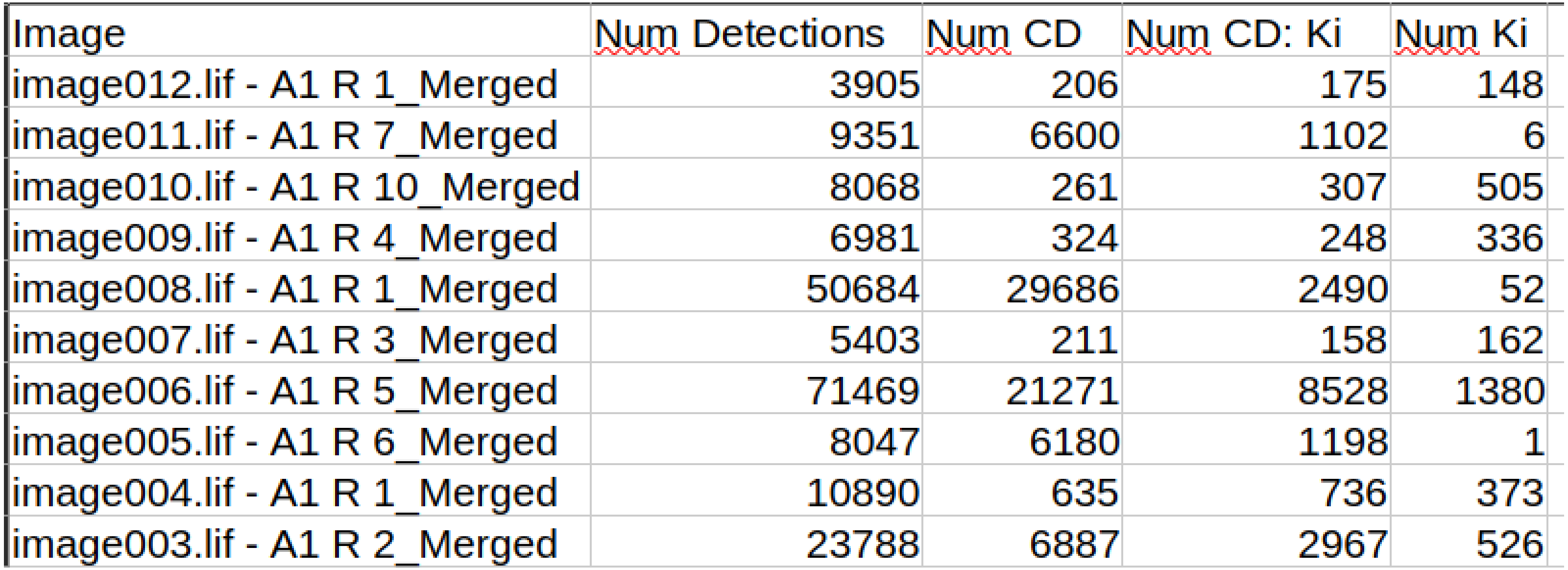


Image	Num Detections	Num CD	Num CD: Ki	Num Ki
image012.lif - A1 R 1_Merged	3905	206	175	148
image011.lif - A1 R 7_Merged	9351	6600	1102	6
image010.lif - A1 R 10_Merged	8068	261	307	505
image009.lif - A1 R 4_Merged	6981	324	248	336
image008.lif - A1 R 1_Merged	50684	29686	2490	52
image007.lif - A1 R 3_Merged	5403	211	158	162
image006.lif - A1 R 5_Merged	71469	21271	8528	1380
image005.lif - A1 R 6_Merged	8047	6180	1198	1
image004.lif - A1 R 1_Merged	10890	635	736	373
image003.lif - A1 R 2_Merged	23788	6887	2967	526

convert_table.groovy

https://github.com/MontpellierRessourcesImagerie/

Total Numbers per class

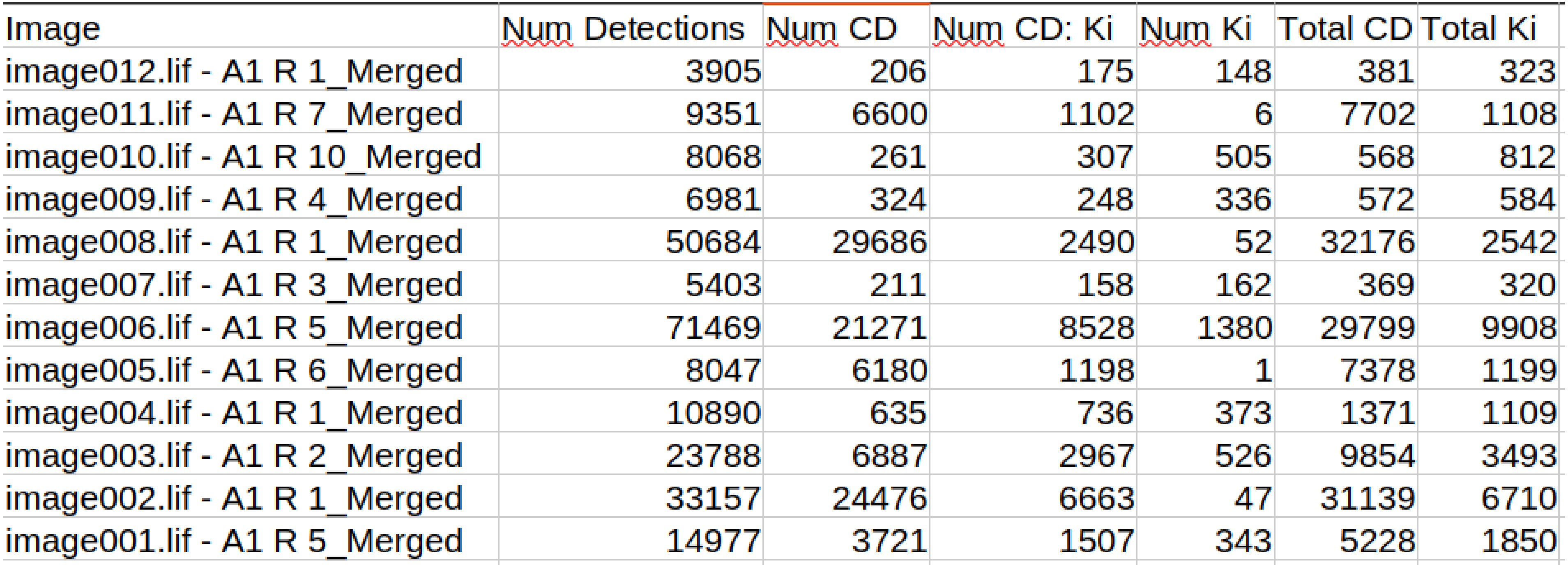


Image	Num Detections	Num CD	Num CD: Ki	Num Ki	Total CD	Total Ki
image012.lif - A1 R 1_Merged	3905	206	175	148	381	323
image011.lif - A1 R 7_Merged	9351	6600	1102	6	7702	1108
image010.lif - A1 R 10_Merged	8068	261	307	505	568	812
image009.lif - A1 R 4_Merged	6981	324	248	336	572	584
image008.lif - A1 R 1_Merged	50684	29686	2490	52	32176	2542
image007.lif - A1 R 3_Merged	5403	211	158	162	369	320
image006.lif - A1 R 5_Merged	71469	21271	8528	1380	29799	9908
image005.lif - A1 R 6_Merged	8047	6180	1198	1	7378	1199
image004.lif - A1 R 1_Merged	10890	635	736	373	1371	1109
image003.lif - A1 R 2_Merged	23788	6887	2967	526	9854	3493
image002.lif - A1 R 1_Merged	33157	24476	6663	47	31139	6710
image001.lif - A1 R 5_Merged	14977	3721	1507	343	5228	1850