**Extended Data 1. Macros used for pre-processing and for morphological segmentation of images using Image-Pro 10.0 software.**

**Macro for Pre-Processing Images**

Public Function PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy() As SimpleScript

 PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy = New SimpleScript

 Dim var1 = "Spatial Cal In situ Focus 20x", spcal1, doc1, image1, doc2, image2, doc3, image3, doc4, image4, image5, doc5, image6, window1, window2, window3, window4, varList1

 With Application.RibbonCommands.SelectRibbonTab(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .TabName = "Home"

 .Run()

 End With

 With Measure.Calibration.SpatialCommands.Define(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(var1, spcal1)

 End With

 With Measure.Calibration.SpatialCommands.SetActive(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Comment = "Spatial Cal In situ Focus 20x: 0.3437 x 0.3437 µm"

 .FilterInput = True

 .Run(spcal1)

 End With

 With Application.DocumentCommands.Active(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc1)

 End With

 With Measure.Calibration.SpatialCommands.Apply(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Comment = "Spatial Cal In situ Focus 20x: 0.3437 x 0.3437 µm"

 .Run(doc1, spcal1)

 End With

 With Application.RibbonCommands.SelectRibbonTab(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .TabName = "Adjust"

 .Run()

 End With

 With Adjust.ImageCommands.Rotate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Orient = Image.OrientType.Rotate180

 .Visible = True

 .Run(doc1, image1)

 End With

 With Application.DocumentCommands.Activate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(image1, doc2)

 End With

 With Adjust.ImageCommands.ExtractChannel(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Channel = 2

 .Interpretation = MediaCy.IQL.Engine.mcInterpretation.mciRGB

 .Run(image1, image2)

 End With

 With Application.DocumentCommands.Activate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(image2, doc3)

 End With

 With Application.RibbonCommands.SelectRibbonTab(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .TabName = "Process"

 .Run()

 End With

 With Adjust.ImageCommands.Duplicate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Name = "Layer 1\_Rotate180CW\_Blue\_BGSubtracted"

 .Visible = True

 .Run(doc3, image3)

 End With

 With Application.DocumentCommands.Activate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(image3, doc4)

 End With

 With Process.BackgroundCommands.Extract(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .BackgroundType = Background.ExtractTypes.Bright

 .FeatureSize = 20

 .Visible = False

 .Run(doc4, image4)

 End With

 With Process.BackgroundCommands.Subtract(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc4, image4, image3)

 End With

 With Adjust.ImageCommands.Duplicate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Name = "Layer 1\_Rotate180CW\_Blue\_BGSubtracted\_BGCorrected"

 .Visible = True

 .Run(doc4, image5)

 End With

 With Application.DocumentCommands.Activate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(image5, doc5)

 End With

 With Process.BackgroundCommands.Extract(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .BackgroundType = Background.ExtractTypes.Bright

 .FeatureSize = 20

 .Visible = False

 .Run(doc5, image6)

 End With

 With Process.BackgroundCommands.Correct(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .BlackLevel = 0R

 .Run(doc5, image6, image5)

 End With

 With Application.DocumentCommands.Activate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc1, doc1)

 End With

 With Application.DocumentCommands.Activate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc5, doc5)

 End With

 With Application.DocumentCommands.Properties(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .DisplayName = "Layer .tif"

 .Run(doc5)

 End With

 With Application.DocumentCommands.Activate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc4, doc4)

 End With

 With Application.WindowCommands.Define(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc4, window1)

 End With

 With Application.WindowCommands.Close(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(window1)

 End With

 With Application.DocumentCommands.Activate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc5, doc5)

 End With

 With Application.DocumentCommands.Activate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc3, doc3)

 End With

 With Application.WindowCommands.Define(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc3, window2)

 End With

 With Application.WindowCommands.Close(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(window2)

 End With

 With Application.DocumentCommands.Activate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc5, doc5)

 End With

 With Application.DocumentCommands.Activate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc2, doc2)

 End With

 With Application.WindowCommands.Define(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc2, window3)

 End With

 With Application.WindowCommands.Close(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(window3)

 End With

 With Application.DocumentCommands.Activate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc5, doc5)

 End With

 With Application.DocumentCommands.Activate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc1, doc1)

 End With

 With Application.WindowCommands.Define(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc1, window4)

 End With

 With Application.WindowCommands.Close(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(window4)

 End With

 With Application.DocumentCommands.Activate(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(doc5, doc5)

 End With

 With Application.WindowCommands.QuickSaveSelectedForAnalysis(PreProYAB\_Calibration\_\_um\_\_\_IN\_SITU\_20XTiffVS\_Naming\_Saving\_Copy)

 .Run(varList1)

 End With

 End Function

**Macro for Segmentation**

Public Function cFOS\_Count\_YAB\_new\_measurements\_for\_INSITU20X() As SimpleScript

 cFOS\_Count\_YAB\_new\_measurements\_for\_INSITU20X = New SimpleScript

 Dim doc1

 With Measure.ThresholdTool.Gadgets.Histogram(cFOS\_Count\_YAB\_new\_measurements\_for\_INSITU20X)

 .CheckState = MediaCy.IQL.Application.McCommand.mcCheckState.Checked

 .Run()

 End With

 With Application.DocumentCommands.Active(cFOS\_Count\_YAB\_new\_measurements\_for\_INSITU20X)

 .Run(doc1)

 End With

 With Measure.MeasurementsCommands.Options(cFOS\_Count\_YAB\_new\_measurements\_for\_INSITU20X)

 .Segmentation.AutoFindPhase = MediaCy.IQL.Features.mcFindPhase.mcfpManual

 .Segmentation.SegmentationType = McMMOptions.mcmmSegmentationType.mcmmstThresholdSegmentation

 .Run(doc1)

 End With

 With Measure.ThresholdToolCommands.Thresholds(cFOS\_Count\_YAB\_new\_measurements\_for\_INSITU20X)

 .AllowOverlap = False

 .Interpretation = eInterpretation.Mono

 .Classes = New System.Collections.Generic.List(Of SegmentationClass)

 .Classes.Add(New SegmentationClass("Parent:(none)",System.Drawing.Color.Blue,New Double(){0R,180R}))

 .Run(doc1)

 End With

 With Measure.Measurements.OptionsCommands.Open(cFOS\_Count\_YAB\_new\_measurements\_for\_INSITU20X)

 .FileName = "C:\Users\alvarezbagnaryg\Desktop\AOW DAB cFos staining\DAB c-Fos detection whole brain 16um\measurement test.iqo"

 .FilterIndex = 1

 .Run(doc1)

 End With

 With Measure.Measurements.HistogramCommands.Options(cFOS\_Count\_YAB\_new\_measurements\_for\_INSITU20X)

 .Measurement = New MeasEntry(eMeasures.RgnArea)

 .Run(doc1)

 End With

 With Measure.MeasurementsCommands.ApplyFilters(cFOS\_Count\_YAB\_new\_measurements\_for\_INSITU20X)

 .Run(doc1)

 End With

 With Measure.MeasurementsCommands.CountByROI(cFOS\_Count\_YAB\_new\_measurements\_for\_INSITU20X)

 .ClassifyByParent = True

 .Run(doc1)

 End With

 With Measure.Measurements.Gadgets.DataTable(cFOS\_Count\_YAB\_new\_measurements\_for\_INSITU20X)

 .CheckState = MediaCy.IQL.Application.McCommand.mcCheckState.Unchecked

 .Run()

 End With

 With Measure.Measurements.Gadgets.DataTable(cFOS\_Count\_YAB\_new\_measurements\_for\_INSITU20X)

 .CheckState = MediaCy.IQL.Application.McCommand.mcCheckState.Checked

 .Run()

 End With

 With Measure.MeasurementsCommands.Selection(cFOS\_Count\_YAB\_new\_measurements\_for\_INSITU20X)

 .SelectionFlag = McMeasurements.enumMMSelTypes.mcmmsfAddWithReset

 .Run(doc1, Nothing)

 End With

 With Measure.Measurements.TableCommands.Options(cFOS\_Count\_YAB\_new\_measurements\_for\_INSITU20X)

 .ShowStatistics = True

 .ShowStatisticsOnly = False

 .Run(doc1)

 End With

 End Function