3 Level Segmentation-

Variables and logs that must be pre-opened at the beginning of the experiment:
blankdatalog (data logging should be paused at beginning of experiment - note that image data will be saved to the da talog; first set of measurements are cytoplasm; second set are nucleus)
hi_value
pulsetime
md_value
pulsetime2
lo_value
pulsetime3

Pulse1 is Correlated to Blue (High) - 800ms recommended (intensely nuclear) - NEED TO DEFINE, variable = hi_valu e
Pulse2 Length is Correlated to Green (Middle) - 200ms recommended (medium nuclear) - NEED TO DEFINE, variabl e = md_value
Pulse3 Length is Correlated to Red (Low) - 50ms recommended (low nuclear) - NEED TO DEFINE, variable = lo_valu e

Note that GFP/Dendra2 image must be taken after miRFP image. So, images should go NIR --> GFP --> RFP (post)

Well = Screen.Status.WellName + "_" + + "Site" + str(Screen.Status.SiteNum)
Power = str(Component.405_Laser_Power.Position) +"% " +"Laser Power"

IF Screen.Status.WaveName="Camera GFP" THEN
Crop GFP, maintaining original image name
name = "Camera GFP"+"_"+"Crop"
1: Create Region()
Position:X/From:X = 0
Position:Y/From:Y = 1
Width/To:X = Image.Width
Height/To:Y = Image.Height-2
2: Image/Plane("Camera GFP")
Image.Name = name
3: Clear All Regions([Current At Start])
Crop NIR, maintaining original image name
name = "Camera NIR"+"_" +"Crop"
4: Create Region()
Position:X/From:X = 0
Position:Y/From:Y = 1
Width/To:X = Image.Width
Height/To:Y = Image.Height-2
5: Image/Plane("Camera NIR")
Image.Name = name
6: Clear All Regions([Current At Start])
Begin segmentation
7: New "LowPass" = Basic Filters("Camera GFP_crop", 5, 5)
8: New "Segmentation" = Count Nuclei(Src=[7: Basic Filters])
9: Close([7: Basic Filters])
10: Threshold Image([8: Count Nuclei], 1, 65535, Inclusive)
11: New "miRFP Binary" = Binarize([8: Count Nuclei]), high = current value, low = current value
12: New "Dilate" = Dilate Image([11: Binary Operations], WITHOUTCLOSING, 1, 3, NODILATECLOSE)
Create image subsets containing only cytoplasm and nucleus pixels; these will be used to calculate stuff.
13: Create Regions Around Objects([12: Dilate Image])
14: New "Cytoplasm" = "Dilate" - [11: Binary Operations] + 0
15: New "NIR_and_cytoplasm" = "Camera NIR_crop" AND [14: Arithmetic]
IMPORTANT STEP: threshold the image such that pixels of 0 are not measured by the region analysis
16: Threshold Image([15: Arithmetic], 1, 65535, Inclusive)
17: New "Nucleus" = Erode Image([11: Binary Operations], 1, 1, KEEPLAST)
18: New "NIR_and_nucleus" = [17: Erode Image] AND "Camera NIR_crop"

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    IMPORTANT STEP: threshold the image such that pixels of 0 are not measured by the region analysis
19: Threshold Image([18: Arithmetic], 1, 65535, Inclusive)
Close un-needed steps (cytoplasm, nucleus, miRFP binary). KEEP Dilate (for regions) and NIR_and_cytoplasm/nucleus
20: Close([11: Binary Operations])
21: Close([14: Arithmetic])
22: Close([17: Erode Image])
Initialize region storage images - use thresholded segmentation image to give a template to shrink regions to fit nuclei
23: Image/Plane([8: Count Nuclei])
24: Rename Image([Last Result], "Reg1_storage")
Reg1 = 0
25: Image/Plane([8: Count Nuclei])
26: Rename Image([Last Result], "Reg2_storage")
Reg2 = 0
27: Rename Image([8: Count Nuclei], "Reg3_storage")
Reg3 = 0
Transfer regions from Dilate to cytoplasm_NIR so measurements can be made
28: Transfer Regions([12: Dilate Image], [15: Arithmetic], ALLREGIONS and CLEARSOURCE)
29: Close([12: Dilate Image])
30: Close("Camera GFP_crop")
31: Close("Camera NIR_crop")
32: Select Image([15: Arithmetic])

Loop through regions in NIR_and_cytoplasm: (1) measure intensitieis, ignoring unthresholded pixels (2) transfer to nucleu
IF Image.NumRegions > 0 THEN
    33: Resume Data Logging()
    FOR Image.ActiveRegion = 1 TO Image.NumRegions STEP 1
        34: Region Measurements([15: Arithmetic], CurrentPlane, Active Region, Milliseconds)
        cytoplasm_avg_intensity = RegionMeasurements.Measurements.AverageIntensity
        35: Transfer Regions([15: Arithmetic], [18: Arithmetic], SELECTEDREGION)
        36: Region Measurements([18: Arithmetic], CurrentPlane, Active Region, Milliseconds)
        nucleus_avg_intensity = RegionMeasurements.Measurements.AverageIntensity
        First, ensure that cell has a readable miRFP value
        Check if ratio is greater than hi value. If it is: transfer region to Segmentation; shrink region to fit; then transfer regi
        IF cytoplasm_avg_intensity > 11000 THEN
            IF nucleus_avg_intensity/cytoplasm_avg_intensity > hi_value THEN
                37: Transfer Regions([18: Arithmetic], "Reg1_storage", SELECTEDREGION)
                38: Shrink Region to Fit("Reg1_storage")
            ELSE
                Check if ratio is greater than md value. If it is, copy region to Reg2_storage. If not, check next threshold.
                IF nucleus_avg_intensity/cytoplasm_avg_intensity > md_value THEN
                    39: Transfer Regions([18: Arithmetic], "Reg2_storage", SELECTEDREGION)
                    40: Shrink Region to Fit("Reg2_storage")
                ELSE
                    Check if ratio is greater than hi value. If it is, copy region to Reg3_storage. If not, do not transfer regior
                    IF nucleus_avg_intensity/cytoplasm_avg_intensity > lo_value THEN
                        41: Transfer Regions([18: Arithmetic], "Reg3_storage", SELECTEDREGION)
                        42: Shrink Region to Fit("Reg3_storage")
                    ELSE
                    END IF
                END IF
            END IF
        ELSE
            IF nucleus_avg_intensity > 11000 THEN
                IF nucleus_avg_intensity/cytoplasm_avg_intensity > hi_value THEN
                    43: Transfer Regions([18: Arithmetic], "Reg1_storage", SELECTEDREGION)
                    44: Shrink Region to Fit("Reg1_storage")
                ELSE
                    Check if ratio is greater than md value. If it is, copy region to Reg2_storage. If not, check next threshol
                    IF nucleus_avg_intensity/cytoplasm_avg_intensity > md_value THEN
                        45: Transfer Regions([18: Arithmetic], "Reg2_storage", SELECTEDREGION)
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                    46: Shrink Region to Fit("Reg2_storage")
                ELSE
                    Check if ratio is greater than hi value. If it is, copy region to Reg3_storage. If not, do not transfer re
                    IF nucleus_avg_intensity/cytoplasm_avg_intensity > lo_value THEN
                        47: Transfer Regions([18: Arithmetic], "Reg3_storage", SELECTEDREGION)
                        48: Shrink Region to Fit("Reg3_storage")
                    ELSE
                    END IF
                END IF
            END IF
        ELSE
        END IF
    END IF
NEXT
49: Pause Data Logging()
ELSE
END IF
50: Close([15: Arithmetic])
51: Close([18: Arithmetic])

Save regions in Reg1_storage, then close it
52: Select Image("Reg1_storage")
IF Image.NumRegions>=1 THEN
    Reg1 = 1
    FOR Image.ActiveRegion = 1 TO Image.NumRegions STEP 1
        Region.ColorBlue = 255
        Region.ColorGreen = 0
        Region.ColorRed = 0
    NEXT
    53: Save Regions("Reg1_storage", "Batch 1 ROI Targeting")
ELSE
    No Regions Present at the Low Threshold
END IF
54: Close("Reg1_storage")

Save regions in Reg2_storage, then close it
55: Select Image("Reg2_storage")
IF Image.NumRegions>=1 THEN
    Reg2 = 1
    FOR Image.ActiveRegion = 1 TO Image.NumRegions STEP 1
        Region.ColorBlue = 0
        Region.ColorGreen = 0
        Region.ColorRed = 255
    NEXT
    56: Save Regions("Reg2_storage", "Batch 2 ROI Targeting")
ELSE
    No Regions Present at the Low Threshold
END IF
57: Close("Reg2_storage")

Save regions in Reg3_storage, then close it
58: Select Image("Reg3_storage")
IF Image.NumRegions>=1 THEN
    Reg3 = 1
    FOR Image.ActiveRegion = 1 TO Image.NumRegions STEP 1
        Region.ColorBlue = 0
        Region.ColorGreen = 255
        Region.ColorRed = 0
    NEXT
    59: Save Regions("Reg3_storage", "Batch 3 ROI Targeting")
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ELSE
    No Regions Present at the Low Threshold
END IF
60: Close("Reg3_storage")
END Segmentation of Image



**********TARGETING FOR MOSAIC -Batch 1 ROI's.....NO NEED TO EDIT SCRIPT/CHANGE BELOW THIS LINE
61: Select Image("Camera NIR")
IF Reg1=1 THEN
    Regions are Saved and loaded to Mosaic for Targeting
    62: Load Regions("Camera NIR", "Batch 1 ROI Targeting")
    current_illumination = Device.Illumination.Setting
    63: Select Illumination("Camera GFP")
    64: Targeted Illumination = Targeted Illumination(Illum setting=Camera GFP, Coord setting=20X APO, Active region, I
        Coordinate system setting = Device.Magnification.Setting
        Mask Exposure Duration [ms] = pulsetime
    65: Delay(MILLISEC)
        Time = pulsetime
    66: Select Illumination("Camera GFP")
        Setting Name = current_Illumination
    67: Clear All Regions("Camera NIR")
ELSE
    Reg1 = 0
    Dont' Utilize the Mosaic on this Field of VIew
END IF



**********TARGETING FOR MOSAIC -Batch 2 ROI's.....NO NEED TO EDIT SCRIPT/CHANGE BELOW THIS LINE
68: Select Image("Camera NIR")
IF Reg2=1 THEN
    Regions are Saved and loaded to Mosaic for Targeting
    69: Load Regions("Camera NIR", "Batch 2 ROI Targeting")
    current_illumination = Device.Illumination.Setting
    70: Select Illumination("Camera GFP")
    71: Targeted Illumination = Targeted Illumination(Illum setting=Camera GFP, Coord setting=20X APO, Active region, I
        Coordinate system setting = Device.Magnification.Setting
        Mask Exposure Duration [ms] = pulsetime2
    72: Delay(MILLISEC)
        Time = pulsetime2
    73: Select Illumination("Camera GFP")
        Setting Name = current_Illumination
    74: Clear All Regions("Camera NIR")
ELSE
    Dont' Utilize the Mosaic on this Field of VIew
END IF



**********TARGETING FOR MOSAIC -Batch 3 ROI's.....NO NEED TO EDIT SCRIPT/CHANGE BELOW THIS LINE
75: Select Image("Camera NIR")
IF Reg3=1 THEN
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        Regions are Saved and loaded to Mosaic for Targeting
        76: Load Regions("Camera NIR", "Batch 3 ROI Targeting")
        current_illumination = Device.Illumination.Setting
        77: Select Illumination("Camera GFP")
        78: Targeted Illumination = Targeted Illumination(Illum setting=Camera GFP, Coord setting=20X APO, Active region, I
            Coordinate system setting = Device.Magnification.Setting
            Mask Exposure Duration [ms] = pulsetime3
        79: Delay(MILLISEC)
            Time = pulsetime3
        80: Select Illumination("Camera GFP")
            Setting Name = current_Illumination
        81: Clear All Regions("Camera NIR")
    ELSE
        Reg1 = 0
        Dont' Utilize the Mosaic on this Field of VIew
    END IF
    82: Select Image("Camera NIR")


ELSE

END IF


Create Overlay Images for Final Display Purposes: Conditions: (1,1,1) (1,1,0) (1,0,1) (1,0,0) (0,1,1) (0,1,0) (0,0,1) (0,0,0)
IF Screen.Status.WaveName="Camera RFP_532x_Post" THEN
    IF Reg1=1 THEN
        IF Reg2=1 THEN
            IF Reg3=1 THEN
                Lo, Mid and High Regions Found  Reg1=1 and Reg 2=1 and Reg3=1
                Reg1=1 and Reg2=1 and  Reg3=1
                #1  (1,1,1)
                (Disabled)83: New "Color Combine" = Color Combine("Camera NIR", "Camera GFP", [None])
                (Disabled)84: Text([Nonexistent command], 10, 10, 0, 255, 0, Arial, Bold, 11, "%Well%     Pulse Time Hi: %pul
                (Disabled)85: Load Regions([Nonexistent command], "Batch 1 ROI Targeting")
                (Disabled)86: Load Regions([Nonexistent command], "Batch 2 ROI Targeting")
                (Disabled)87: Load Regions([Nonexistent command], "Batch 3 ROI Targeting")
                (Disabled)88: Add to "Target Regions Overlay" = As Displayed([Nonexistent command], Entire image)
                (Disabled)89: Close([Nonexistent command])
            ELSE
                #2(1,1,0)
                (Disabled)90: New "Color Combine" = Color Combine("Camera NIR", "Camera GFP", [None])
                (Disabled)91: Text([Nonexistent command], 10, 10, 0, 255, 0, Arial, Bold, 11, "%Well%     Pulse Time Hi: %pul
                (Disabled)92: Load Regions([Nonexistent command], "Batch 1 ROI Targeting")
                (Disabled)93: Load Regions([Nonexistent command], "Batch 2 ROI Targeting")
                (Disabled)94: Add to "Target Regions Overlay" = As Displayed([Nonexistent command], Entire image)
                (Disabled)95: Close([Nonexistent command])
            END IF
        ELSE
            Reg1=1 and Reg 2=0 Reg3=1
            IF Reg3=1 THEN
                #3 (1,0,1)
                (Disabled)96: New "Color Combine" = Color Combine("Camera NIR", "Camera GFP", [None])
                (Disabled)97: Text([Nonexistent command], 10, 10, 0, 255, 0, Arial, Bold, 11, "%Well%     Pulse Time Hi: %pul
                (Disabled)98: Load Regions([Nonexistent command], "Batch 1 ROI Targeting")
                (Disabled)99: Load Regions([Nonexistent command], "Batch 3 ROI Targeting")
                (Disabled)100: Add to "Target Regions Overlay" = As Displayed([Nonexistent command], Entire image)
                (Disabled)101: Close([Nonexistent command])
            ELSE
                #4 (1,0,0)
```

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            (Disabled)102: New "Color Combine" = Color Combine("Camera NIR", "Camera GFP", [None])
            (Disabled)103: Text([Nonexistent command], 10, 10, 0, 255, 0, Arial, Bold, 11, "%Well%     Pulse Time Hi: %p
            (Disabled)104: Load Regions([Nonexistent command], "Batch 1 ROI Targeting")
            (Disabled)105: Add to "Target Regions Overlay" = As Displayed([Nonexistent command], Entire image)
            (Disabled)106: Close([Nonexistent command])
        END IF
    END IF
ELSE
    Reg1=0 and Reg2=1
    IF Reg2=1 THEN
        Reg1=0 and Reg2=1 and Reg3=1
        IF Reg3=1 THEN
            #5 (0,1,1)
            (Disabled)107: New "Color Combine" = Color Combine("Camera NIR", "Camera GFP", [None])
            (Disabled)108: Load Regions([Nonexistent command], "Batch 2 ROI Targeting")
            (Disabled)109: Load Regions([Nonexistent command], "Batch 3 ROI Targeting")
            (Disabled)110: Text([Nonexistent command], 10, 10, 0, 255, 0, Arial, Bold, 11, "%Well%     Pulse Time Hi: %p
            (Disabled)111: Add to "Target Regions Overlay" = As Displayed([Nonexistent command], Entire image)
            (Disabled)112: Close([Nonexistent command])
        ELSE
            #6 (0,1,0)
            (Disabled)113: New "Color Combine" = Color Combine("Camera NIR", "Camera GFP", [None])
            (Disabled)114: Load Regions([Nonexistent command], "Batch 2 ROI Targeting")
            (Disabled)115: Text([Nonexistent command], 10, 10, 0, 255, 0, Arial, Bold, 11, "%Well%     Pulse Time Hi: %p
            (Disabled)116: Add to "Target Regions Overlay" = As Displayed([Nonexistent command], Entire image)
            (Disabled)117: Close([Nonexistent command])
        END IF
    ELSE
        IF Reg3=1 THEN
            #7 (0,0,1)
            (Disabled)118: New "Color Combine" = Color Combine("Camera NIR", "Camera GFP", [None])
            (Disabled)119: Load Regions([Nonexistent command], "Batch 3 ROI Targeting")
            (Disabled)120: Text([Nonexistent command], 10, 10, 0, 255, 0, Arial, Bold, 11, "%Well%     Pulse Time Hi: %p
            (Disabled)121: Add to "Target Regions Overlay" = As Displayed([Nonexistent command], Entire image)
            (Disabled)122: Close([Nonexistent command])
        ELSE
            #8 (0,0,0)
            (Disabled)123: New "Color Combine" = Color Combine("Camera NIR", "Camera GFP", [None])
            (Disabled)124: Text([Nonexistent command], 10, 10, 0, 255, 0, Arial, Bold, 11, "%Well%     Pulse Time Hi: %p
            (Disabled)125: Add to "Target Regions Overlay" = As Displayed([Nonexistent command], Entire image)
            (Disabled)126: Close([Nonexistent command])
        END IF
    END IF
END IF
END IF
```