### Neun Area and Intensity Protocol Notes from Daniel’s Demonstration

### (Updated 6/07/2024)

**Important to remember: Always save your ROI on ROI manager (to do this press t) this is so that you do not have to remeasure when you need to recheck**

1. **Measuring Hippocampi for DG, Hilus, and GCL**
2. Open the NeuN stain (purple/magenta)
   1. Image -> Type -> 16 bit
   2. Now B&W/gray, go to Image -> Lookup Tables -> Magenta
   3. If channels are combined do Image -> colors -> split channels and find the needed channel, change the channel color if needed
3. Start with the DG, we need the area and intensity
4. Go to Image -> Adjust -> Brightness/Contrast (top option)
   1. Increase the maximum (left direction) to make the fluorescence more saturated/intense, this is only so we can see the molecular layer which we include for the DG ROI.
5. DG ROI: ROI is the molecular layer (follow line/blood vessel holes), should end/align with the blades of the DG and follow along above the ventricles
6. Analyze -> measure (crtl + m)
   1. Area is our Area and Mean is our intensity from our Measure Table
7. Doing the Hilus and GCL, reset and automatic/to get original brightness and contrast
8. We need to set our threshold, we do this by creating a region without any NeuN positive cells
   1. Select the polygon feature and draw the area on the control side, this will be in the molecular layer since there is a low level of cells here. The area can be small or large, but there must not be any positive cells. Its fluorescence will mess with the threshold, to check for this increase the intensity of fluorescence.
   2. Be sure to go to Analyze -> Set Measurement -> Check off Standard Deviation if you haven’t already, then measure
   3. To calculate the threshold: Formula for Threshold: (7 x std.) + mean intensity
   4. Image -> Adjust -> Threshold, set and edit the lower threshold with what was calculated
   5. Do the regular ROI for the Hilus and GCL
9. Two different tasks for CA3: measuring the area and measuring the intensity
10. **CA3 Area**
    1. First create an ROI for the CA3, from inside the Hilus to the area before it starts to curve
    2. We want the area, more specifically the length to be very similar to the other side
       1. To do this, go to Edit -> Selection -> Scale
          1. Set X to -1 and Y to 1 to flip the ROI
11. **CA3 intensity \*\*last updated 9/3/2024**
    1. Starting at the CA3 tip, create an entire length of 600 microns
       1. I used the segmented lines, to save to ROI you must make it complete (connect the last box to the first, you will have to reverse/overlap to complete it)
       2. If you notice that your length does not equal 600 microns, it could be that it is set to inches by default.
          1. You can change this by going to Analyze > Set Scale
             1. Slide Scanner (Taken at Microscopy Core):

Distance in pixels: 3.0758

Known distance: 1.00

Pixel aspect ratio: 1.000

Unit of length: micron (um)

* + - * 1. You should see that the scale says: 3.0758 pixels/micron
        2. Keyence

Same information above, but the distance in pixels should ve 1.5 pixels/micron ratio

* + - * 1. The type of scanner can be figured out by the date it the image was taken or by testing the length on the image and looking at the measurement in relation to the CA3 area
  1. Duplicate this length twice to create an up region and a down line
     1. Duplicating can be done by selecting the original and
     2. Rename one as (original numbers - up)
     3. Rename the other as (original numbers - down)
        1. Note: I have labeled the original as “CA3 Intensity Original”, and the others as “CA3 - Up” and “CA3 - Down” also noting whether it is ipsi or contra
  2. Select the Up version and go to translate (located in the ROI Manager, select More>> and towards the end)
     1. UP: X: 0 and Y:-40
  3. Select the Down version and go to translate
     1. DOWN: X: 0 and Y:+40
  4. Draw a polygon connecting the corners
     1. Do this by selecting the show all option on the ROI manager
  5. Once a polygon has been made:
     1. Check if centered, if not attempt to modify if able to
     2. Rename the polygon as the final
        1. Note: I have labeled them as, “Final CA3 Intensity Polygon”
     3. Measure and find the mean, the mean is our intensity
        1. We only use the area to check how similar our measurement for the polygon size, in theory they should be the same if not very similar

**Original CA3 measurement instructions: \*now updated above: 2/13/24**

1. CA3 will be different
   1. First create an ROI for the CA3, from inside the Hilus to the area before it starts to curve
   2. We want the area, more specifically the length to be very similar to the other side
      1. To do this, go to Edit -> Selection -> Scale
         1. Set X to -1 and Y to 1 to flip the ROI
2. We want to see the intensity of the CA3, so we take the diameter
   1. Starting on the CA3 part in the Hilus, stop at the blades and make a note of the length, we want to duplicate this to determine its end
   2. From this start the box/rectangle going up, outline the CA3 in Hilus- going underneath the GCL’s blades and back around until the box is complete
   3. This is now the new intensity of CA3

**(Image example here)**