I.N.T.E.N.S.I.T.Y. Analyzer v2.0

(Immunohistochemistry Neural Tissue Engineering-lab Noise and Signal Illumination Threshold Yaba Analyzer)

READ ME/Manual

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Version Update Information

V 2.0 11/25/2015

- Redefined '*Normalized Intensity*' to '*Signal-to-Noise Intensity Ratio (SNIR)*' to better reflect calculations and avoid confusion (see **Core Mathematical Concept**, p1).
- Added FFT option to flatten images before processing without control images.

V 1.1 08/28/2015

- Prevented INTENSITY from including "tissue area" inside the "probe footprint area" into the area of the first bin in planar and multishank mode. (in V1.0, tissue in the probe footprint area was intentionally included to average tissue pull out and processing related deformation into the calculation of the first bin)

About

This is an open source MATLAB file package. If any of the script (in-part or whole) is used, please acknowledge Takashi D.Y. Kozai and Zhannetta V. Gugel by citing the paper “Chronic tissue response to carboxymethyl cellulose based dissolvable insertion needle for ultra-small neural probes.” *Biomaterials*. 2014, 35(34), 9255-9268[5]
Thank you.

From the Manuscript

Kozai, Gugel, Li, Gilgunn, Khilwani, Ozdoganlar, Fedder, Weber, Cui. “Chronic tissue response to carboxymethyl cellulose based dissolvable insertion needle for ultra-small neural probes.” Biomaterials. 2014

“A custom MATLAB script was written to perform intensity-based radial analysis for activity/density dependent fluorescent markers (OX-42/GFAP/IgG/NF/Laminin). For the analysis, images were compared to control data 500-700 μm away from any insertion sites. Due to the change in size over time and irregular shape of the CMC shuttle wound ‘hole’, it was necessary to adjust the quantitative method from previously established methods to prevent low intensities from the implant ‘hole’ from artificially decreasing the average of fluorescence markers in the actual tissue (Fig.S2). In order to prevent holes in the tissue (such as major blood vessels and probe tracks) from artificially reducing the average activity-dependent fluorescence, background noise intensity threshold was calculated from corresponding control images. To calculate the background noise intensity threshold, pixels with intensity greater than one standard deviation dimmer than mean pixel intensity were considered “signal” and removed from the calculation. The threshold was then determined by calculating the pixel intensity of one standard deviation below the mean of the remaining pixel intensities. Bins with intensity values dimmer than average intensities of the control images were considered tissue “holes.” Using MATLAB, the center of the probe track was identified on each image and the electrode tip was modeled with a 1 μm radius, after which the script generated masks of concentric rings every 10 μm for 320 μm. The average gray scale intensity for all pixels above the background noise intensity threshold in each 10 μm ring was calculated, normalized against the background, and plotted as a function of distance. Data were averaged for each probe type and time point, and then reported as mean and standard error.

For NeuN, a custom MATLAB script was modified to generate masks of concentric rings every 25 μm for 325 μm from a user-defined lesion site. For NeuN control images, 1 concentric ring with a radius of 325 μm was generated from the midpoint of the images. The bins corresponding to the lower and upper bounds of each mask were superimposed on all NeuN images. Within each mask, pixels with intensity values greater than 1 were included in the analysis. Because care was taken during image acquisition to optimize image offset, pixel values less than 1 were indicative of tissue “holes.” A threshold was not utilized to allow for tissue area calculation by retaining pixels corresponding to background (NeuN- regions) and signal (NeuN+ cells). An array per mask was created by assigning a value of 1 for each remaining pixel. To calculate tissue area, the sum of the amount of remaining pixels was converted into square microns using the confocal objective resolution at 20X magnification (0.62 μm/pixel). The tissue area per radial bin was exported for each image. Two counters quantified the total NeuN+ cells in each radial bin using ImageJ. Each person identified and maintained a counting metric throughout all NeuN quantification. NeuN density (neuron count per tissue area) was calculated and normalized by the NeuN density of corresponding control images. As previously, data were averaged for each probe type and time point, and the mean and standard error were plotted as a function of distance.”

Core Mathematical Concept

The average intensity for all pixels above the background noise intensity threshold in each bin was calculated and normalized against the background to calculate the Signal-to-Noise Intensity Ratio (SNIR) in each bin as follows;

$$SNIR = \frac{AvgI_{>T}}{AvgN} \quad (1)$$

where $AvgI_{>T}$ is the mean Intensity of all pixels above the noise threshold (>*T*) in each bin, and *AvgN* is the mean noise floor intensity. **This means, SNIR=1 represents the noise floor. Therefore, it is expected that the SNIR does not asymptote to 1 unless there is no staining signal in the corresponding bin.** Data were averaged for each implant type and time point, and then reported as mean ± standard error.

This concept, although slightly different, is similar to single-unit SNAR, multi-unit SNFRR, and LFP SNLPR [7].

READ ME

Image Acquisition

For this analysis package, it is critical that the images are acquired correctly. Laser(light source) and PMT(camera settings) need to be optimized such that the threshold is set low enough to detect holes in the tissue (blood vessels, probe track, tears) and background is high enough to detect some reflectance/refractance/auto-fluorescence in all tissue regions. Laser power and PMT gain should also be set such that neither the experimental or control tissue ever saturates pixels (Figure 1). Importantly, when naming your new folder to save all generated data, avoid invalid strings such as '**?**' '**<**' '**>**' '****' '**/**' '*****' '**:**' '**|**' (http://support.microsoft.com/kb/177506). (If an Invalid file name was accidentally used, intensity_CrashRecovery.m can be used to recover the last analyzed image)

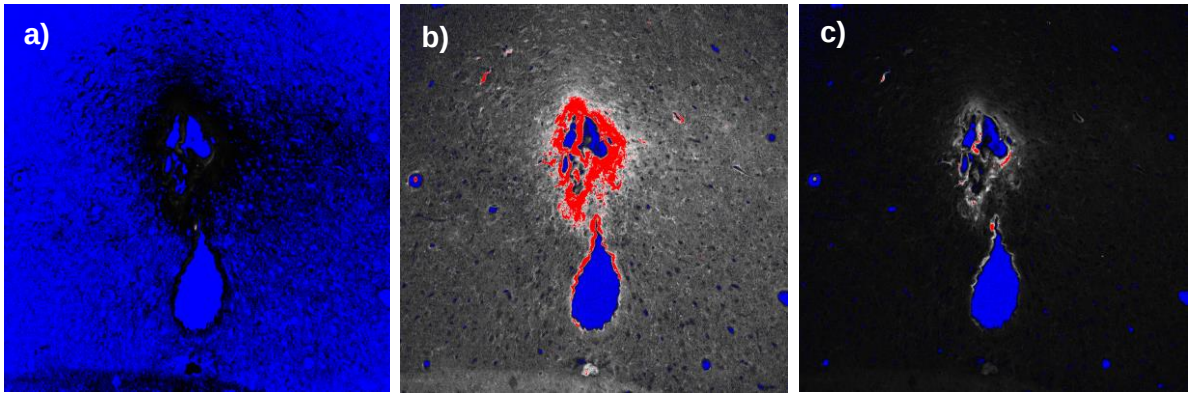


Figure 1: Example images shown in HiLo demonstrate varied PMT laser settings. *Blue* represents pixels that are below the camera detection threshold while *red* represents pixels that are oversaturated to the maximum intensity value of the bit depth. a) Detection threshold is set too low for background tissue to be acquired by the camera. Images with such settings will result in errors during background calculation and normalization. **b)** Pixels near the RoI are saturated thereby disrupting the intensity distribution in the image. **c)** Ideal balance of PMT laser settings showing background threshold set to detect holes while retaining image background and minimal high intensity pixels near the RoI for optimal pixel intensity distribution.

Control images must be taken with the same Laser, PMT, threshold, gain, etc settings with minimal photobleaching between images. Control images should be taken far enough away from the tissue reaction of the experimental region of interest (RoI), but close enough such that the cortical region and depth of the image region are the same. If the same region in the contralateral hemisphere is used, care should be taken to ensure that the sections were not at an angle and that the control and experimental RoI are from the same depths.

File Organization (Multichannel/Multicolor Images)

In general, it is preferred if you use 12 bit or 16+ bit images. Images should be monochromic. For multichannel imaging, separate black and white images (TIFs) should be organized into folders with filenames ending with distinct and consistent channel numbers. (i.e. "*_001.tif", "*_002.tif", "*_003.tif", "*_004.tif"). If Images were acquired as RGB color or CYMK color, imageJ should be used to separate the color into individual black and white images.

Features and Additions

Planar Probe Geometries

Planar probes have rectangular cross-sectional area, not elliptical. Therefore rectangular profiles should be used to characterize planar probes. In these probes, the recording sites are generally at the surface of the probes, not the center, therefore bin distances are automatically calculated from the surface of the probe profile. This generally leads to rectangle with rounded edges.

Tissue Holes, Tear, and Pullouts

Holes and tissue pullout is factored into the analysis. If the hole is large, such that there is no tissue in the bins immediately adjacent to the probe, a '0' is placed into the bin mean. When averaged across animals, standard error and statistical tests will consider bins with '0's as NO DATA and are removed from the N. (for example, if tissue is analyzed for 5 animals, but in 1 animals this is a 0 in the first bin, Std Error and statistical analysis will consider the first bin to have N=4, while the remaining bins will be calculated with N=5). This also eliminates any bias and loss of information which can result from averaging line profiles.

Fast Fourier-Transform to Flatten Images.

Most confocal images do not move the XY stage as it is scanning the tissue. Instead, typically a mirror is used to bend the laser to reach different XY regions of the sample without moving the sample or objective. As a result, the sample directly below the objective lens becomes slightly brighter than the corners of the sample (parts of the sample that are farthest from the object). Because fluorescent signal from the corners of the image have to travel further and at an acute angle to the objective lens surface compared to fluorescent signal at the center of the image, the corners of the image appear substantially darker than the center (even in control images). Fast Fourier-Transform to normalize the image is one mathematical method to correct for this aberration post-hoc. (FFT using control images are not supported at this time).

Steps, Functionality and Visualization Overview

Getting Started
To run intensity analyzer, open Intensity_Main.m with your current version of MATLAB (Mathworks). Prepare parameters for analysis including **1)** electrode dimensions (length and width of probe cross section for *planar* and *multi* analyses; electrode radius for *radial* analysis) **2)** desired bin size in microns **3)** number of bins for analysis **4)** scale conversion (microns/pixel) of the image.

A menu will pop-up asking *New File?* The **New file** option is to be selected if the code has not been run previously, if new analysis parameters will be used, or if the user wishes to manually input previously used parameters. **Load file** will locate an Excel file containing previously used parameters for input steps **1-4** above and apply the same parameters for analysis. Use **Plot files** to plot previously analyzed data. This option prompts the user to select a folder containing results from Intensity_Main.m. Data in the folder must have been analyzed using the same parameters. A function will be called to plot the mean SNIR profile of each imaging channel within the folder. After a selection has been made, the user chooses a destination for a new folder in which all output figures and Excel files generated during analysis will be saved. The user then inputs a name for the new folder.

The following steps assume the New file option was chosen during the previous step.

Analysis Selection
A second menu will pop-up containing the analysis options including *Radial Probe*, *Planar Probe*, *Planar (Multishank)*, *Planar Probe (Single side)*, *Planar (Single side Multishank)*. A detailed overview of each method will be presented below. At this point, the user will input parameters **1-4** as described above.

Background Parameters

The code allows the user to choose how background intensity is calculated in input step **5.** An input number of '0' allows the user to select a control image for background calculation. The mean background intensity will be used to set a threshold for the experimental image. An input number greater than '0' will not prompt the user to select a control image. Instead, the background intensity will be calculated from the corners of the experimental image. For example, if a numerical input of '5' was typed by the user, the code will calculate the intensity in the corner of the image which will be 5% of each image dimension in size. The mean intensity of the corners will be set as the background intensity and threshold for analysis of the same image. Step **6** sets the standard deviation multiplier to determine background intensity. For example, if the input is '1', all pixels that are 1 standard deviation greater than the mean intensity of the control image or image corners are removed from noise calculation. The remaining pixels will be used to calculate the background threshold. Increasing (6), decreases the estimation for signal and increases noise pixels for noisefloor calculation. (6) should not be less than 1. For **7)** the user must input the value for the standard deviation from the background to find the image threshold. All pixels that are below the threshold are removed from analysis (considered as holes). For (6) and (7), the user should select the best values in terms of σ (standard deviation) from an understanding in signal & systems and statistical probability theory. **8)** If (5) had a value greater than zero, and option will be available to use FFT to flatten image before processing. Input '1' to flatten the image or '0' to proceed without flattening.

Selecting Images for Analysis

Select a folder containing multiple channels (stains) per image (Figure 2a, Figure 3). Images should be black and white images. If input step **5** was a numerical '0', then the user will need to select a control folder that corresponds to the selected image folder (Figure 2b). Each channel within the experimental folder must correspond to each channel within the control folder (for example: Channel 1 must be DAPI, Channel 2 must be Alexa 488, Channel 3 must be Alexa 568, etc.). Note that if the number of images in the control folder are not equal to that of the experimental folder, there will be an error message in the MATLAB command prompt. The code will loop through each image in the folder and apply the same input parameters in steps **1-4** to each channel.

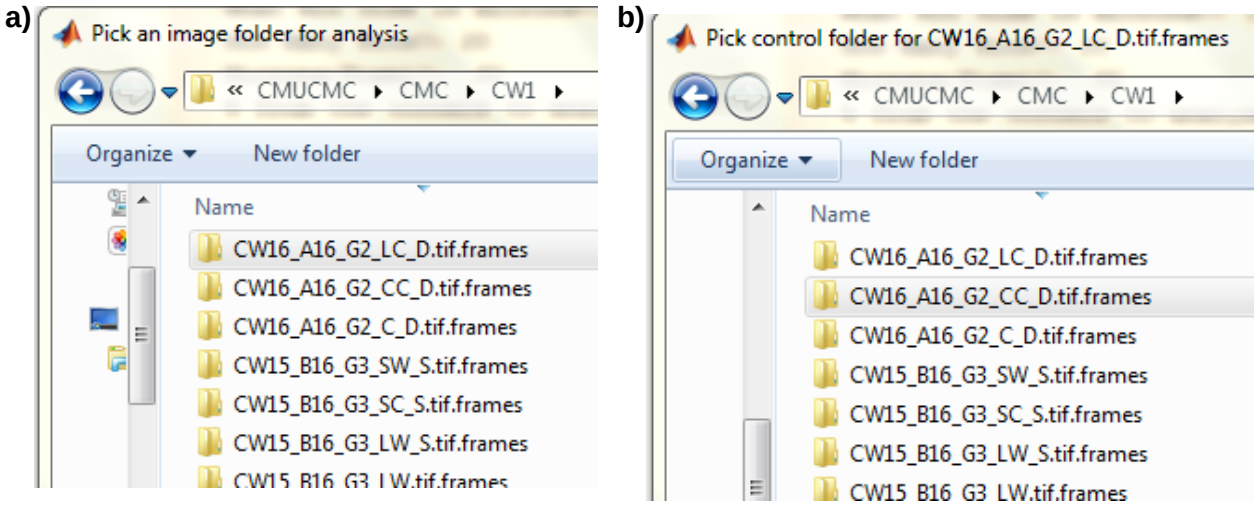


Figure 2: a) Screen-shot of the experimental image folder selection. The user chooses a folder containing multiple channels per imaging session. **b) Screen-shot of control folder selection for corresponding experimental image.** The dialog box displays the experimental image folder selected as a hint to the user. Note this step will be skipped if input step **5** was a numerical input greater than '0'.

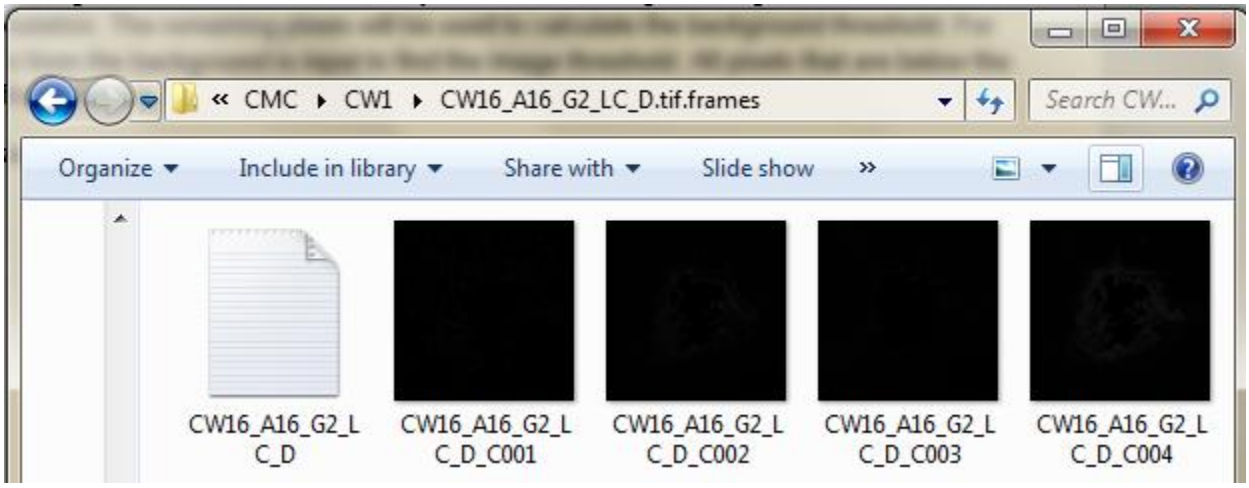


Figure 3: Channels within Image folder. The experimental and control folders may have multiple imaging channels. The code will load each channel and corresponding control one-by-one to perform intensity-based analysis with the same binning and electrode parameters. The background and threshold will be recalculated for each channel using a corresponding control. If the corners of the experimental image are used to find the background, the mean corner background intensity calculation will be repeated for every channel as well. Here, a .txt file with imaging parameters is saved in the folder, but only .tif files are recognized and loaded into MATLAB.

Selecting the RoI

After the background has been calculated and displayed on the command prompt, the experimental image fill be loaded and displayed on a figure. From here, the user must choose the center of the RoI from where the analysis will begin and subsequent bins will be plotted (Figure 4). The code will save the coordinates of the chosen RoI and apply the same center point to each channel with an image folder for analysis. Therefore, the user must choose the RoI once per image folder.

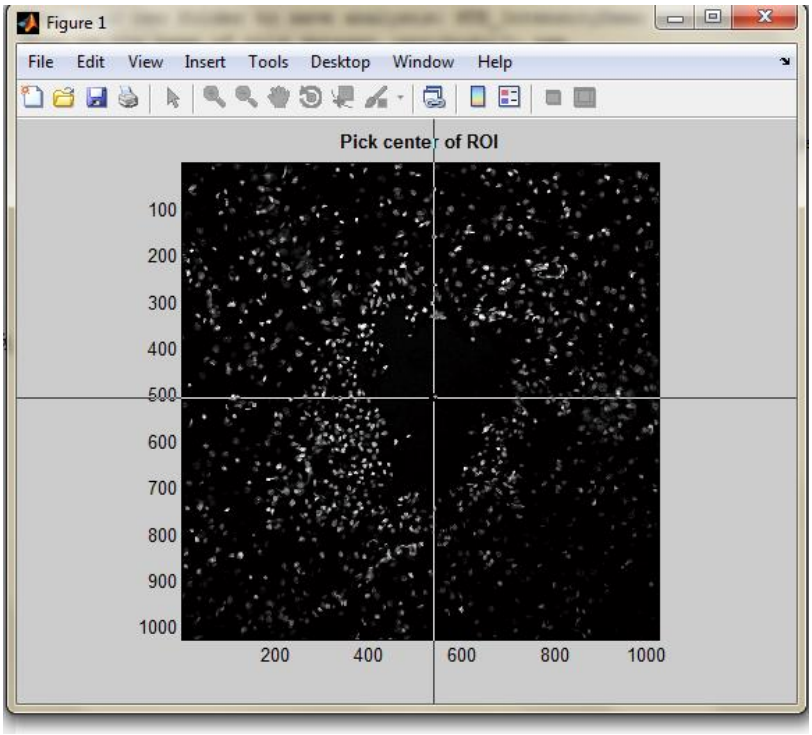


Figure 4: Screen-shot of loaded image folder and RoI selection step. The user selects the center of the image from where intensity analysis and bins will begin. The same center point of channel 1 will be applied to any subsequent channels within the same image folder.

Output and Interface

The figure used to select the RoI (Figure 4) is called to plot bins using user-specified parameters under a given analysis method. Examples of bins using methods available in Intensity_Main.m are shown below (Figure 5). As the script calculates mean SNIR per bin, a bin will be plotted on the experimental image. Binned images are useful for reporting cell number (i.e. Neuronal, glial, etc) as a function of distance from the implant. Furthermore, our code quantifies the mean bin area to allow for cell density quantification (see Figure 7 for output area example). Additional binning metrics are available including single sided planar and single sided multi analysis (examples shown in Figure 5f,g).

The second output figure is the main interface (Figure 6). Briefly, the figure displays the SNIR profile as a function of distance from the implant. The mask applied to the image (parameterized by the bin size and image analysis method chosen) is shown as well as the background pixel distribution, control image used for background calculation and experimental image after threshold was applied. In Figure 6, the analysis method was *Planar Probe* (rectangle with rounded edges).

Quantitative output data is saved to an Excel file for each channel (appended by _chanNUM, where NUM is the channel number). The data stored in the file contains the inner bin location, outer bin location (both in microns), normalized mean SNIR, mean bin area (μm^2), threshold value used for the experimental image, % of the pixels that were removed from analysis (% filtered), average intensity of the background, and standard deviation of the background pixels. Each column is conveniently labeled, and the original filename is used to name the Excel file. See the Excel interface in Figure 7.

All outputs are named and saved into the user-specified folder automatically. After saving, the figures are closed to avoid over-burdening the RAM if subsequent analysis is to be performed. The user can then select to

continue analyzing with the same metrics, choose an alternate analysis method with new metrics or end analysis. Upon ending analysis, it is possible to plot the results using the program.

Visualizing the Output

To plot the results or previous analysis, select a folder that contains images analyzed with the same method and parameters. The function *probe_plot.m* will ask for the total number of channels that have been analyzed. This information can be found by observing the appended filenames (_chanNUMtot, where NUMtot is the total number of channels analyzed in the folder). The mean and standard error of the SNIR among all Excel files of corresponding channels will be calculated and plotted as a function of distance (mean bin location). A sample output figure is shown in Figure 8. The data used to generate the figure is saved in a new Excel file with each channel saved on a separate sheet (Figure 9).

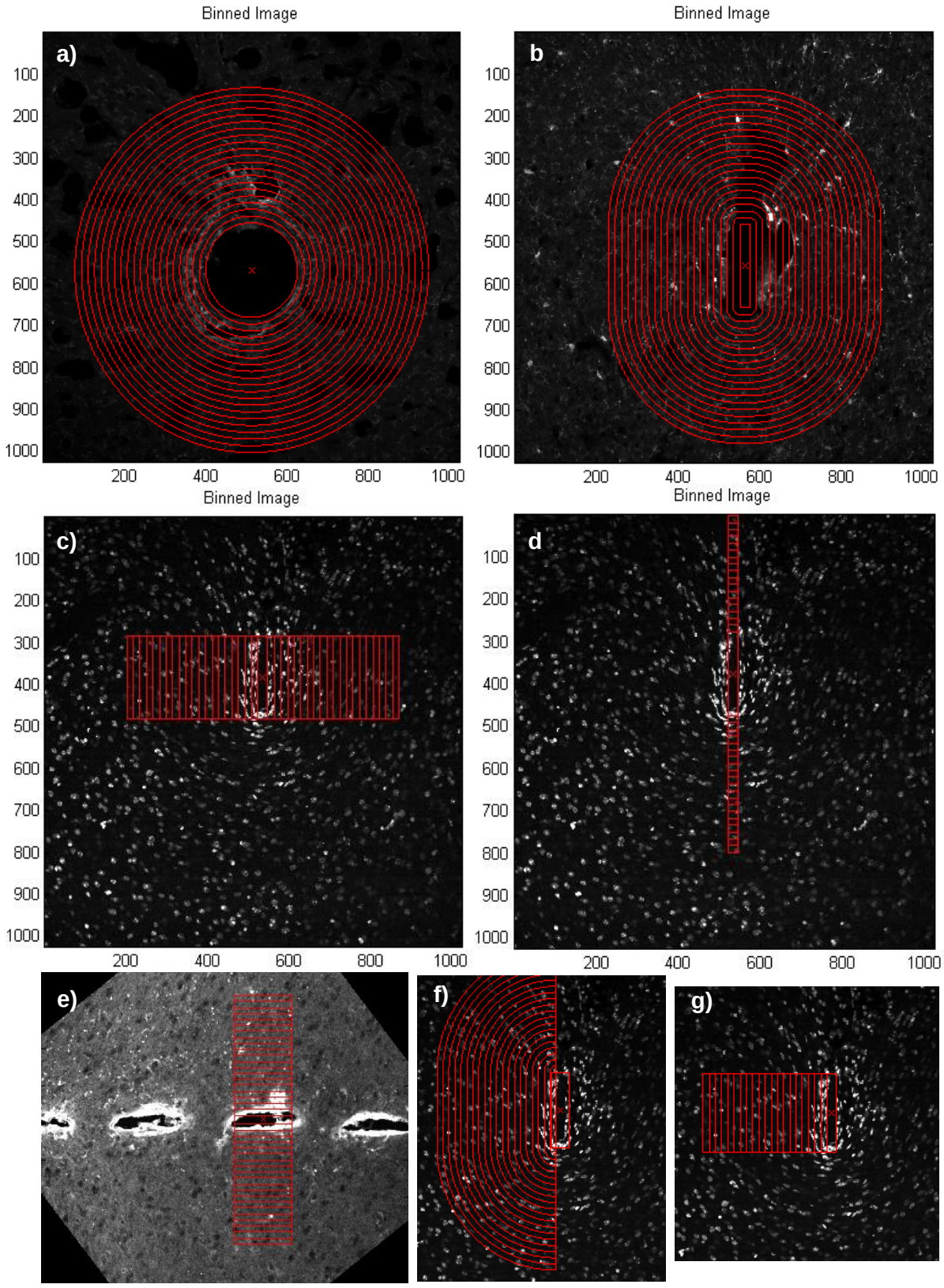


Figure 5: Methods for Image Binning a) *Radial* analysis with concentric circles useful for round probes. **b)** *Planar* analysis generates rectangles with rounded edges. Useful for single shank probes with rectangular cross sectional geometry. **c-d)** *Planar (Multishank)* analysis in the X and Y-direction, respectively. User can select direction for analysis. **e)** Example application of *Planar (Multishank)* analysis. **f)** *Planar (Single side)* shown in Left- direction. **g)** *Planar (Single side Multishank)* shown in Left-direction.

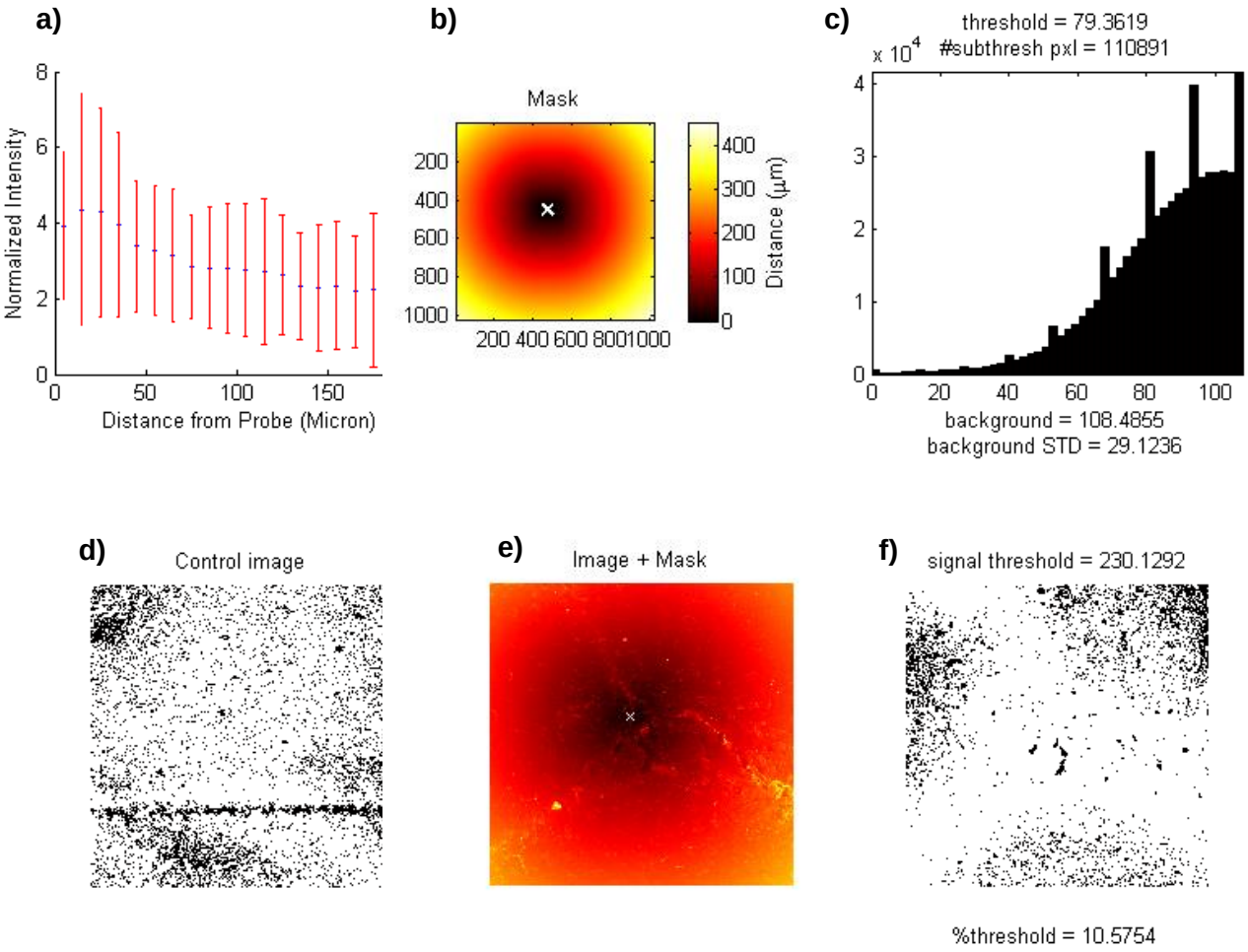
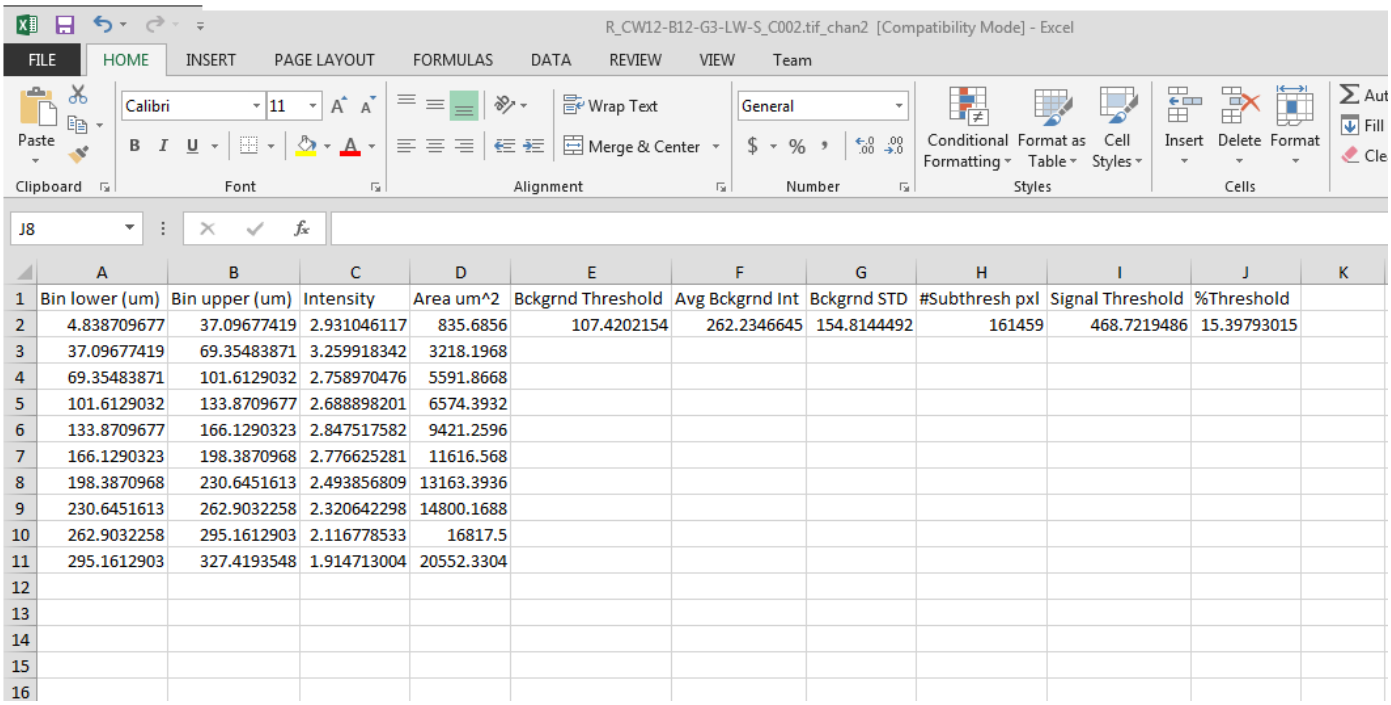


Figure 6: Output Figure of Main Interface. a) Intensity Profile- Mean and standard deviation of the pixel SNIR per bin (distance from Probe) is shown. SNIR values are normalized to the noisefloor by the control image in (d). **b) Binning Mask-** Mask applied to the experimental image. White 'x' shows the user-specified center of the RoI. Colormap displays the distance from the RoI. **c) Background Pixels-** Distribution of pixels used to calculate background noise (in this example, distribution is calculated from the control image). Text above subplot is the threshold for background calculation from the control image and the number of pixels below threshold (used towards noise calculation). Text below subplot is the average intensity and the standard deviation of the control image (or image corners if input step **5** was greater than '0'). **d) Image for Background Calculation-** Black and white control image (or image used to calculate background noise) showing pixels with intensity values above the background intensity (white) and below background, or noise, (black). **e) Image + Mask-** Experimental image with mask superimposed to show regions of intensity calculations. White 'x' shows center of the RoI. **f) Image for Analysis-** Black and white experimental image with pixels above background threshold (white) and below threshold (black). Text above image is the signal threshold used and below image is the percent of pixels below the threshold. White pixels above the threshold were included in intensity analysis.



	A	B	C	D	E	F	G	H	I	J	K
1	Bin lower (um)	Bin upper (um)	Intensity	Area um^2	Bckgrnd Threshold	Avg Bckgrnd Int	Bckgrnd STD	#Subthresh pxl	Signal Threshold	%Threshold	
2	4.838709677	37.09677419	2.931046117	835.6856	107.4202154	262.2346645	154.8144492	161459	468.7219486	15.39793015	
3	37.09677419	69.35483871	3.259918342	3218.1968							
4	69.35483871	101.6129032	2.758970476	5591.8668							
5	101.6129032	133.8709677	2.688898201	6574.3932							
6	133.8709677	166.1290323	2.847517582	9421.2596							
7	166.1290323	198.3870968	2.776625281	11616.568							
8	198.3870968	230.6451613	2.493856809	13163.3936							
9	230.6451613	262.9032258	2.320642298	14800.1688							
10	262.9032258	295.1612903	2.116778533	16817.5							
11	295.1612903	327.4193548	1.914713004	20552.3304							
12											
13											
14											
15											
16											

Figure 7: Excel File Output. Columns *A* and *B* contain the lower and upper bounds (in µm) of each successive bin, respectively. Column *C* is the mean SNIR within the corresponding bin, while Column *D* is the mean area of each bin. The background threshold pixel value (used to separate background from noise) is in Column *E*. The average background intensity and standard deviation (of the control image or from the experimental image corners) is in Column *F* and Column *G*, respectively. The number of sub-threshold pixels in the experimental image, the intensity threshold for analysis and the percent of pixels removed from intensity calculation (below threshold) is stored in Columns *H*, *I*, and *J*, respectively.

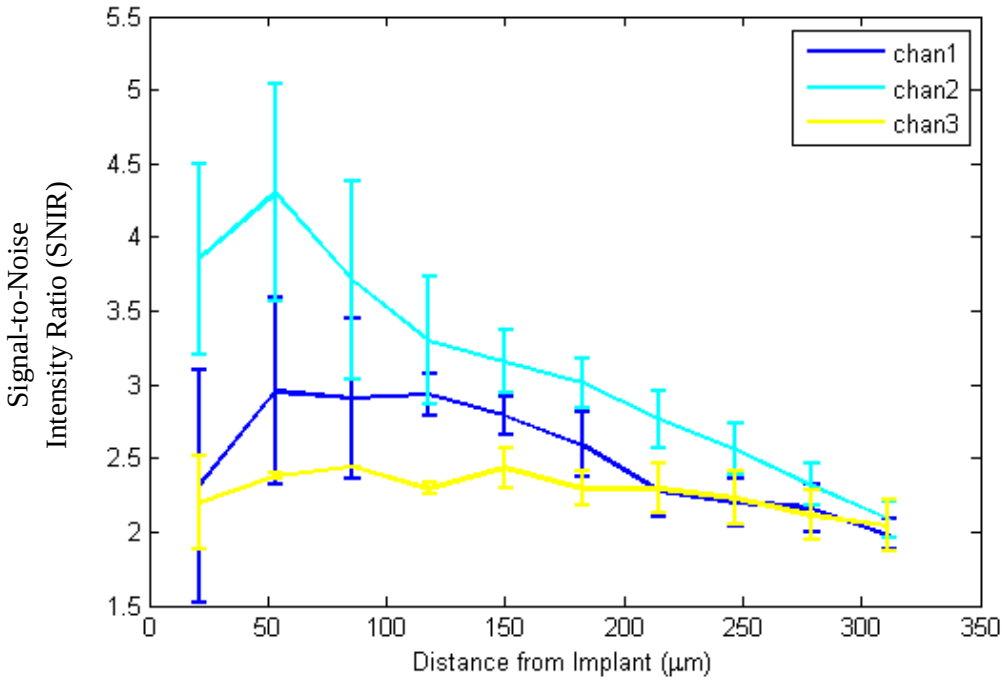
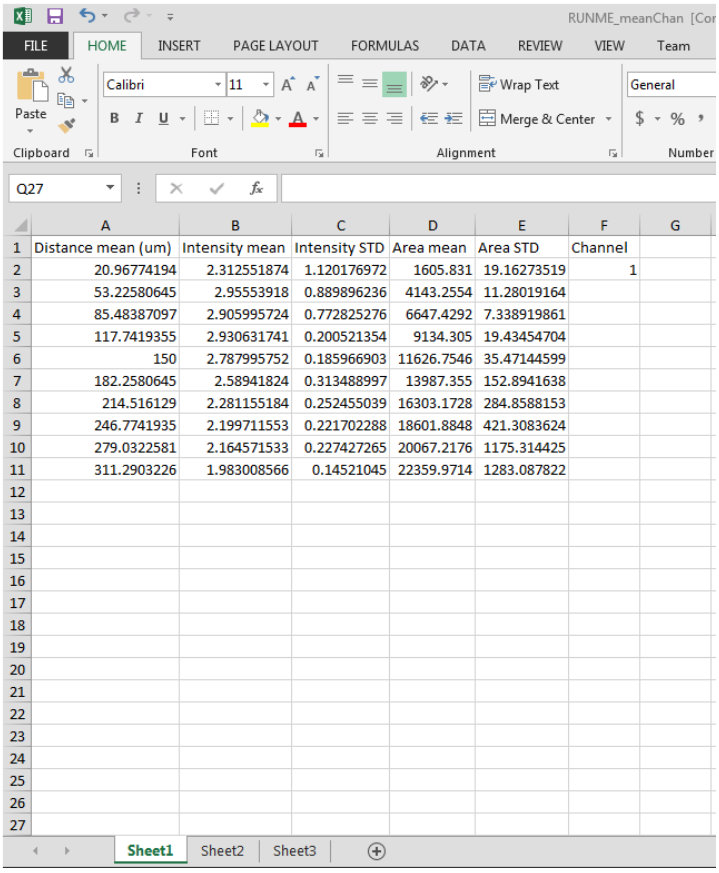


Figure 8: Output of plotting function in Intensity_Main.m. Figure shows mean and standard deviation of intensity profiles for channels 1-3 (blue, cyan, and yellow, respectively) plotted as a function of distance from the implant. The figure shows the data obtained from *Radial analysis* method. The electrode was modeled with a 3µm radius and binning parameters consisted of 20 micron sized bins, 10 bins total and a 0.62 microns/pixel conversion factor. A control image was used to calculate background intensity and the STD background multiplier and the STD from the background were both set to 1. Optional plotting function includes area profile (silenced in code).



	A	B	C	D	E	F	G
1	Distance mean (um)	Intensity mean	Intensity STD	Area mean	Area STD	Channel	
2	20.96774194	2.312551874	1.120176972	1605.831	19.16273519	1	
3	53.22580645	2.95553918	0.889896236	4143.2554	11.28019164		
4	85.48387097	2.905995724	0.772825276	6647.4292	7.338919861		
5	117.7419355	2.930631741	0.200521354	9134.305	19.43454704		
6	150	2.787995752	0.185966903	11626.7546	35.47144599		
7	182.2580645	2.58941824	0.313488997	13987.355	152.8941638		
8	214.516129	2.281155184	0.252455039	16303.1728	284.8588153		
9	246.7741935	2.199711553	0.221702288	18601.8848	421.3083624		
10	279.0322581	2.164571533	0.227427265	20067.2176	1175.314425		
11	311.2903226	1.983008566	0.14521045	22359.9714	1283.087822		

Figure 9: Excel output of data displayed in Figure 8. The mean bin location data, mean and standard error of the intensity, mean and standard error of the bin area as well as channel identifier are saved in an Excel file. File is named according to the folder in which the raw data is stored. Sheet 1-Sheet 3 include data for channels 1-3, respectively.

Background

Planar devices generally have rectangular cross-sections. The original planar silicon probes from the University of Michigan/Center for Neural Communications Technology were made with Boron Diffusion (1988~2000s)[8]. This gave the device a semi-elliptical profile (Fig. 10a). However, it should be noted that the critical surface (the surface with the electrode sites) are completely flat and not semi-elliptical. Therefore, it would be more accurate to model the tissue reaction from the flat electrode site mounted surface of the silicon probe instead of modeling the tissue reaction as an ellipse.

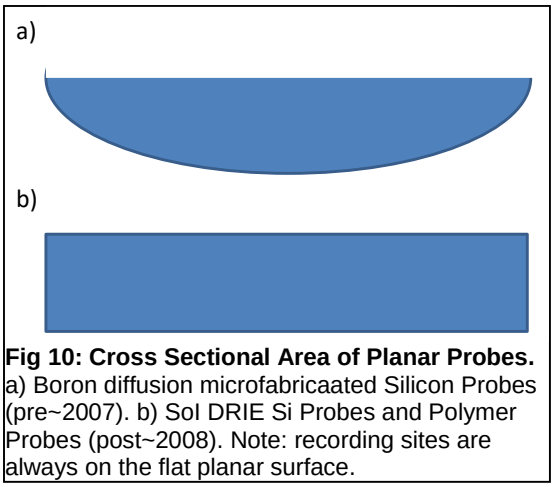


Fig 10: Cross Sectional Area of Planar Probes. a) Boron diffusion microfabricaated Silicon Probes (pre~2007). b) SoI DRIE Si Probes and Polymer Probes (post~2008). Note: recording sites are always on the flat planar surface.

Furthermore, in 2008 NeuroNexus Technologies as well as other research groups and commercial manufacturers around that time switched fabrication methods to Silicon-on-Insulator technology with Deep Reactive Ion Etching[9] (Fig 10b). As a result, the devices now have a rectangular cross-sectional area (Fig. 11) (see also [10]). It is also worth mentioning that most polymer probes have rectangular cross-sectional areas. Therefore, for planar probes, we automated previously established methods of quantifying equal distance bins from the surface of the probe footprint (Fig. 11) [3, 6].

This method provides a much more accurate representation of the probe-tissue interface than arbitrarily defining ellipses at the implant site (Fig. 12-14).

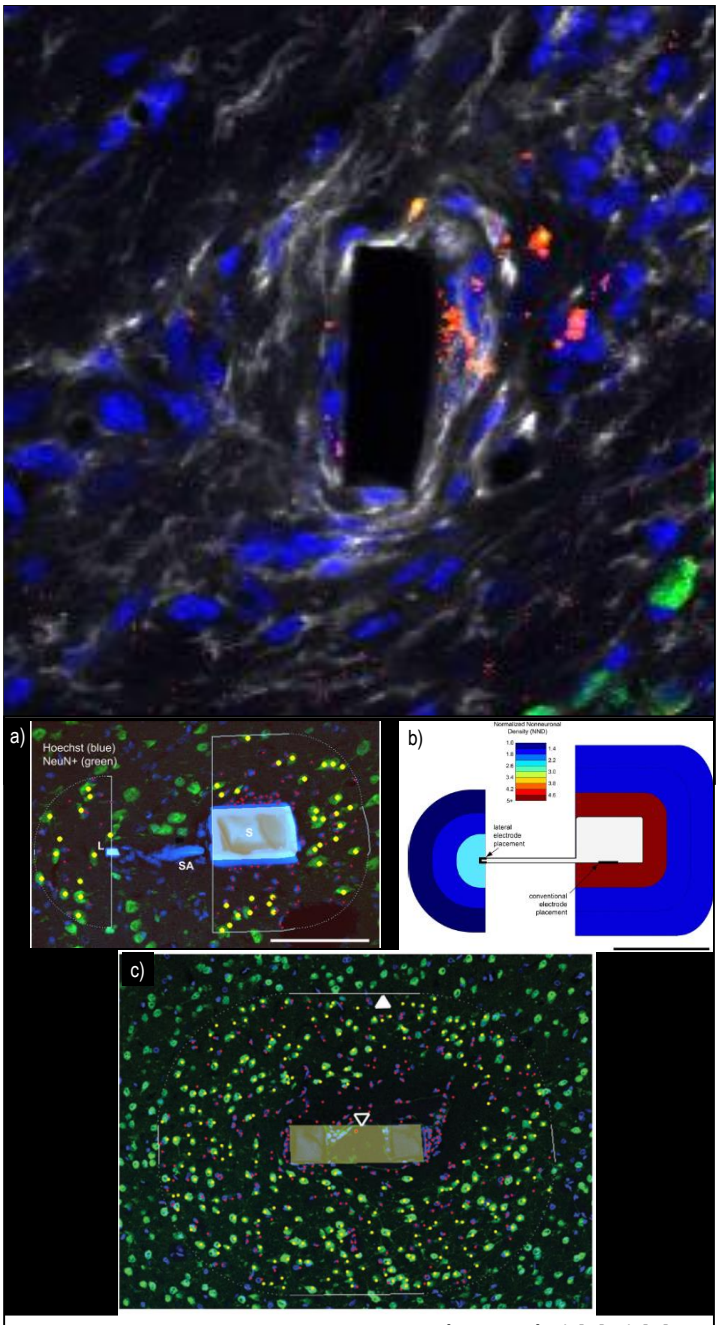


Figure 11: Previous established Binning (manual) a) [3] b) [6]

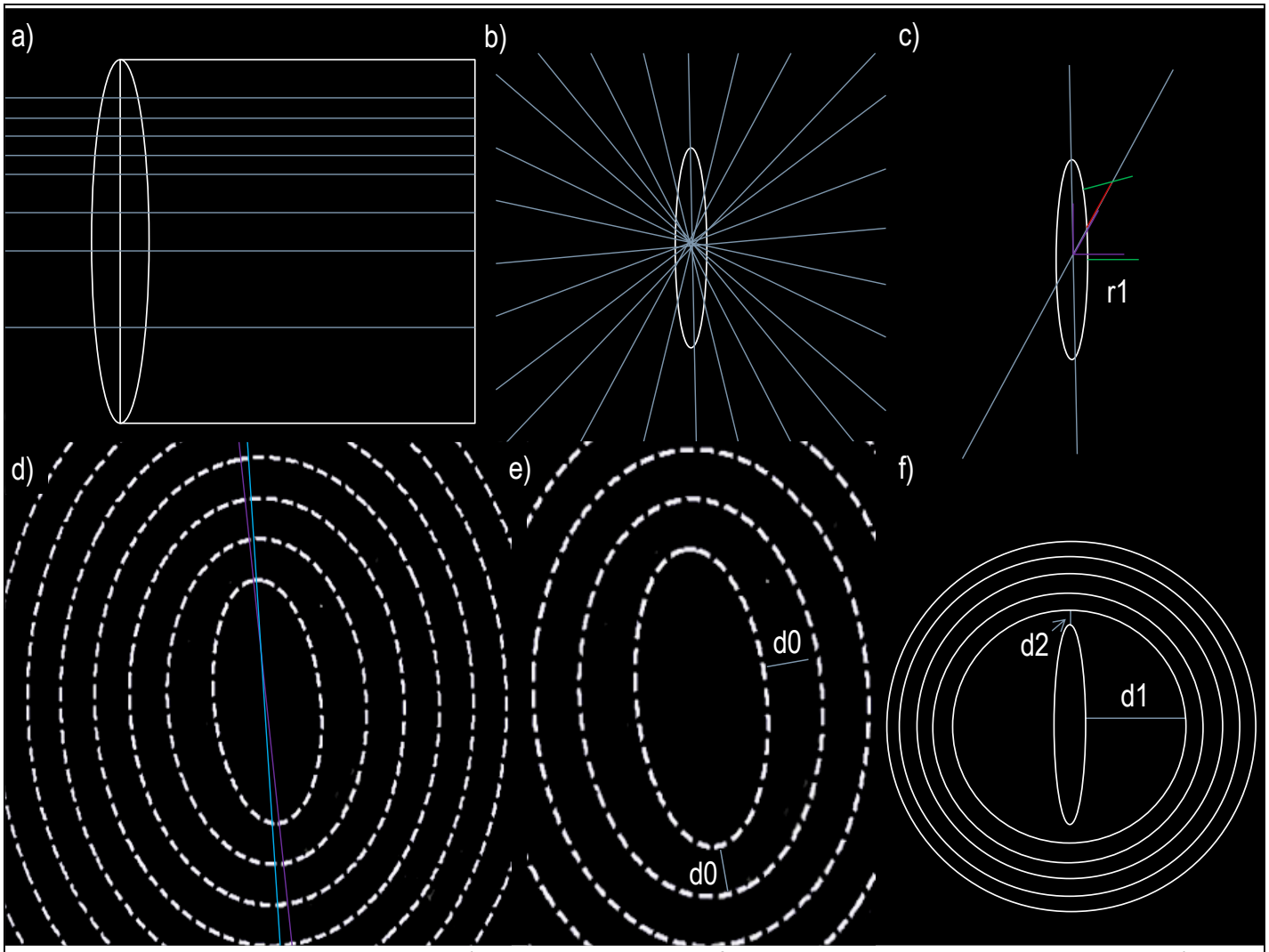


Figure 12: Previously published methods. a) Arbitrary placement of lines and ellipse can bias measurements. b) Similar to (a), except c) shows that because distances are not normal to the ellipse perimeter, it can misrepresent distances from the probe. (purple, red, green lines have the same distance, r1). Purple: from the center of the probe, Red: from the surface of the probe parallel to the radial line profiles, Green: radially projecting normal from the surface perimeter. d-f) previously published method. d) ellipses are off axis, e) Ellipses are not similar (different major and minor axis ratio). Blue line, d0, have the same length, but do not equally extend out to the next bin. f) Newer version of (d-e) but the differences are much more apparent. d1≠d2. Each of these methods can introduce substantial bias in the analyzed data

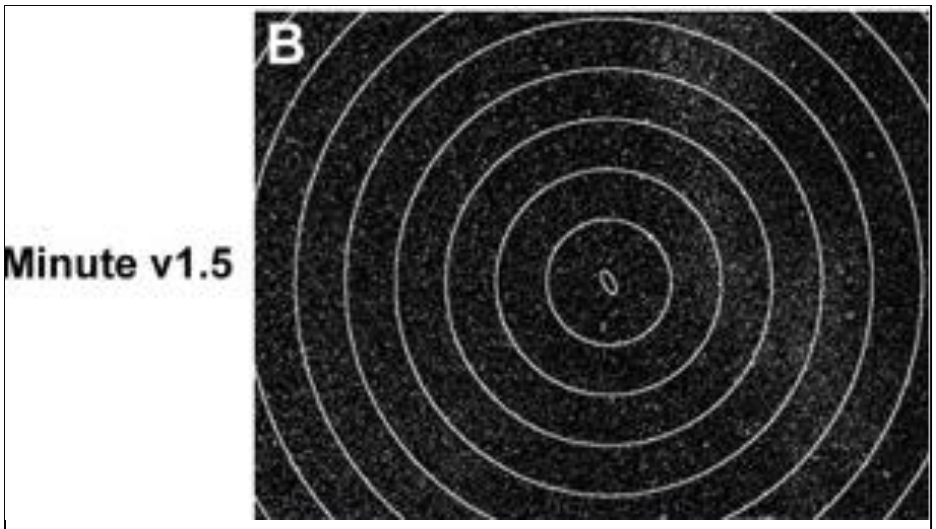


Figure 13: Previously Published Method (same as Fig. 5f)[2]

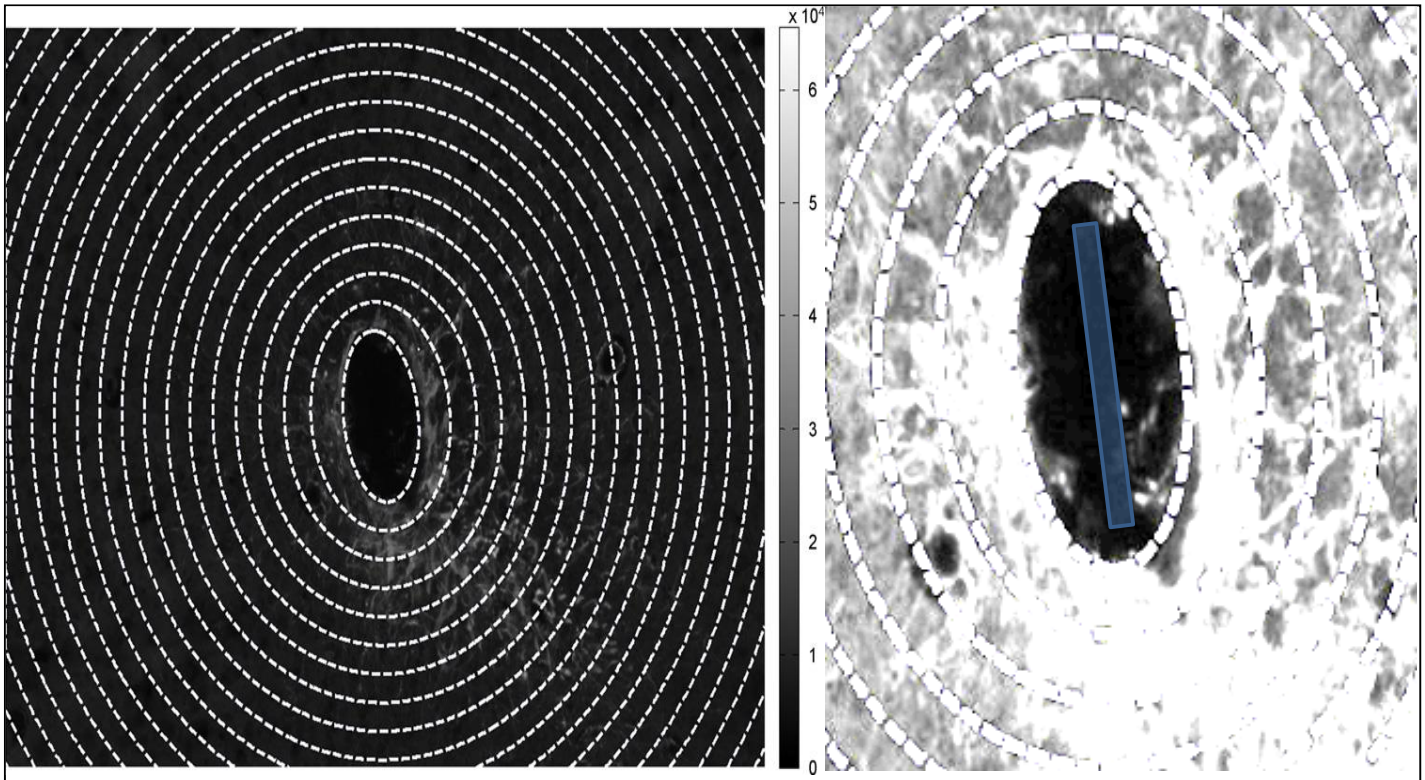


Figure 14 Biases from arbitrarily defined ellipses[1]. a) Arbitrary placement of lines and ellipses. b) The arbitrarily defined probe ellipse shows that there is tissue at the center of the "probe track" that is being removed from analysis. The actual probe track is likely to be the blue probe track overlay. Previous studies have shown that (b) more likely follows this expression pattern around chronically implanted electrodes, particularly for GFAP. The non-GFAP internal sheath is likely ED1+ glial cells (Fig. 15), however this information lost due to the arbitrarily defined ellipse.

Tissue Holes

Tissue holes from probe-tissue adhesion related explantation, large blood vessels, and tissue freezing artifacts can also bias analysis results. There is no tissue there, which means there are no cells there, so there is not activity. The image instead registers SNIR values below the baseline from low-level autofluroescence and/or light reflection/refraction. The low SNIR values artificially decrease the average, thereby biasing the actual activity levels in the tissue.

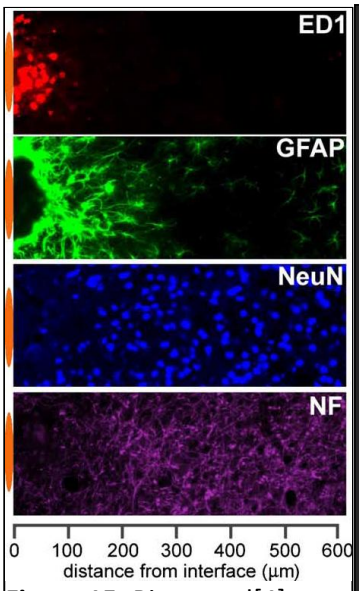


Figure 15: Biran et al[4]. ED1+ cells occupy tissue space inside of GFAP scar.

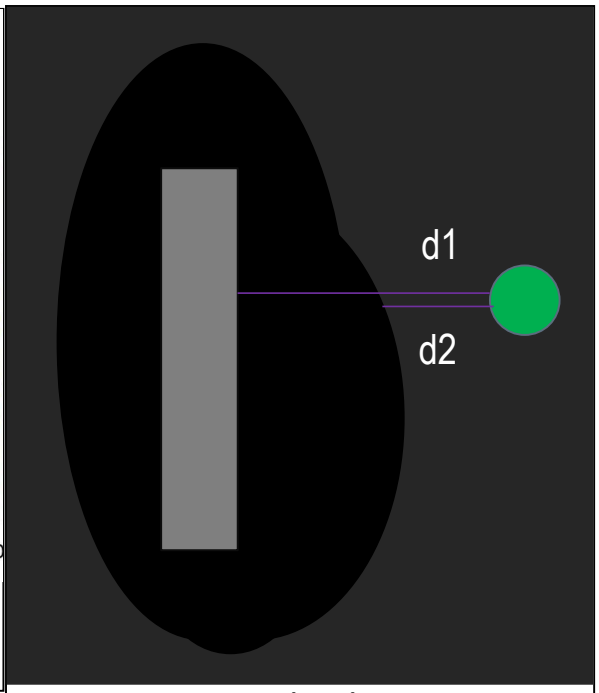


Figure 16: Tissue holes (blue). The distance to the nearest neuron (Green) is d1, NOT d2. Therefore, it makes sense to model the probe cross-sectional area, and not the perimeter of the hole.

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Acknowledgements

This is an open source MATLAB file package. If any of the script (in-part or whole) is used, please acknowledge Takashi D.Y. Kozai and Zhannetta Gugel and cite the paper “Chronic tissue response to carboxymethyl cellulose based dissolvable insertion needle for ultra-small neural probes.” *Biomaterials*. 2014, 35(34), 9255-9268[5]
Thank you.