**Detecting GPCR Complexes in Postmortem Human Brain with Proximity Ligation Assay and a Bayesian Classifier**

**Supplemental Methods and Materials**

*Processing of human brain samples*

The study was approved by the Institutional Review Board (IRB) of New York State Psychiatric Institute (NYSPI, Protocol 6477R). Human brain specimens (Suppl. Table 1) were from autopsies at the Institute for Forensic Medicine, Skopje, Macedonia. The cerebral hemispheres were sliced coronally at 2 cm intervals. Slices from the left hemisphere were placed in home-made cassettes and fixed in 10% formalin in phosphate buffer (PB, pH 7.4) at room temperature for 5 days, then rinsed in tap water and transferred to phosphate buffered saline (PBS) with 0.02% sodium azide at 4°C. The fixed slices were examined by a neuropathologist who dissected a standard series of blocks, which were dehydrated and infiltrated with paraffin in a vacuum infiltration processor [[1](#_ENREF_1)]. Paraffin-embedded blocks of the rostral left striatum, including ventral putamen (Ptm), ventral caudate (Cdt), and nucleus accumbens (NAcc), were used in this study. Sections were cut at a thickness of 6 μm at the Core Facility, Department of Pathology, the Herbert Irving Comprehensive Cancer Center (HICCC), Columbia University Medical Center.

*Specimen preparation from mouse brains*

All mice were handled in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals. Experimental protocol (NYSPI1388) was approved by the Institutional Animal Care and Use Committee (IACUC) at NYSPI. Whole brains were collected from adult mice (3 months old). The mice were deeply anesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg) mix for all the following tissue collection. Transcardial perfusion was performed with 4% paraformaldehyde (PFA) in PBS (pH 7.4). Brains were removed and post-fixed in 4% PFA overnight at 4 oC. Coronal sections (30 μm) of unembedded fixed tissue were generated with a vibrating blade on a LeicaVT 1200 slicer and stored in PBS with 0.02% sodium azide for up to one week until PLA. When appropriate, mouse brains were collected without transcardial perfusion, and fixed in 4% PFA for 4 days at 4 oC. When indicated, the fixed brains were trimmed, dehydrated, embedded in paraffin, and sectioned (6 μm) at the Core Facility, Department of Pathology, HICCC, Columbia University Medical Center. To collect fresh frozen samples, brains were removed and snap frozen in chilled 2-methylbutane (−20°C), and stored at −80°C [[2](#_ENREF_2)]. Frozen sections (20 μm) were prepared with a Cryostat (Leica CM3050S), mounted on positively charged glass slides (Fisher Scientific), air-dried, and fixed with 4% PFA in PBS for 10 mins before PLA.

*PLA on Brain sections*

PLA on sections from mouse brains fixed by transcardial perfusion was performed in 24-well plates according to the published protocol for free-floating sections [[3](#_ENREF_3)]. PLA on snap-frozen and paraffin-embedded brain tissue was performed on sections mounted on positively charged slides (Fisher Scientific). The paraffin sections for immunohistochemistry (IHC) and PLA were deparaffinized in xylene, rehydrated in serial denatured ethanol baths (Fisherbrand™, HistoPrep™), and rinsed twice briefly in 0.1 M Tris-buffer with 0.9% saline (TBS). Antigen retrieval was performed by boiling brain sections in 10 mM sodium citrate buffer (pH 6.5) for 6 min (with a 5-min-interval in the microwave after the first 3 min and addition of buffer to compensate for evaporation). The sections for PLA brightfield (PLA-BF) were quenched with 1% H2O2 for 30 min to inactivate endogenous peroxidase. After brief rinses with TBS containing 0.1% Triton X-100 (TBS-T), sections were incubated with blocking buffer (Duolink blocking buffer for PLA) at room temperature for 1 hour, and then with primary antibodies diluted in the Duolink antibody dilution buffer at 4 oC overnight. Immunofluorescent PLA (PLA-FL) (red) and brightfield PLA (PLA-BF) were performed with the Duolink PLA Fluorescence protocol and Duolink BrightField protocol (Sigma) according to the manufacturer’s manual. In this study we performed both single-recognition PLA (single PLA), which allows detection of a single antigen with only one primary antibody, and dual-recognition PLA (dual PLA), which detects the D2R-A2AR complex with two primary antibodies (A detailed protocol is available online).

Luxol fast blue and cresyl violet (LFB/CV) staining of the human serial paraffin sections were performed according to standard protocol at the HICCC to outline the sub-territories of the striatum.

*Systematic Random Sampling*

Systematic random sampling for human brain whole slide scanned images was performed according to the following protocol. The size and the number of sampling areas and counting loci are shown in suppl. Table 2.

1. Outline the brain region of interest (ROI) for each sample: Open a virtual whole slide image (scn file) of LFB/CV staining (SF. 1A-C) in the SCN400 viewer (Leica, version 2.2), zoom to a full view of the image that fits the screen, and export this full view image (SF. 1A-C) as a tiff file with the “ROI image export" function. Open the full view image in Photoshop (Adobe), create a new layer (a separate outline layer), draw an outline of the brain section and its sub-territories (i.e. ROI and landmarks to orientate the brain section) on the new layer, and print the outline layer on a transparent film without scaling (SF. 1D).
2. Create a sampling grid: In Adobe Illustrator, create a grid image of the same size as the outline image. Print the grid on a transparent film without scaling. This printout is the grid for sampling (SF. 1E). Overlap the films of outline and sampling grid. The brain ROIs are thus divided into a number of sampling areas (SF. 1F). Each grid cell corresponds to one sampling area in a full view image of the brain section (SF. 1G, blue).
3. Select and mark the counting loci: Mark a location (the upper left corner in this study) inside each cell on the sampling grid as the counting locus (SF. 1G, purple). It can be anywhere inside the cell but should be consistent across all cells.
4. Sampling: Open a virtual whole slide image (scn file) of PLA in SCN400 viewer, and zoom in to get a full view image (~0.4x in this study) that fits the screen. Overlap and mount the outline film and the sampling grid film on the screen to cover the entire ROI. Place the mouse indicator at one counting locus marked in step 3, scroll the middle button of the mouse to zoom the image to 40X (as he slides were scanned with a 40x lens). Right click to export the image at 40x as a tiff file with the “ROI image export" function. This is a full magnified counting image (SF. 1H) corresponding to a counting locus (SF. 1G, purple), and will be analyzed as a representative of the sampling area (SF. 1G, blue). Repeat this for all sampling areas inside the ROI. A counting locus is only analyzed when it is located inside the ROI. Note: Do not move the mouse until the zooming is completed.
5. Examine the quality of the images and exclude unfocused or oversaturated images because they will not be analyzed correctly. In this study, images with white matter areas (over 20%) were also excluded.

*Quantification with Particle Analysis* (Image J) and *Spot Detector* (ICY)

All RGB images were first imported to Image J (*Batch*)[[4](#_ENREF_4)], transformed to 8-bit images and the contrast was increased 3% (*Enhance Contrast*). To use *Particle* *Analysis,* an automated (~170) or manual (110) threshold was applied to all images and then the *Particle* *size* was set at *1-5 um2*, which was based on manual measurements of PLA puncta from both single and dual PLA assays. The *Overlay* of analyzed particles (PLA puncta) in *ROI manager* was saved and imported to the original images for post-analysis review.

For quantification with ICY (version 1.9.10.0) [[5](#_ENREF_5)], the RGB images were first transformed to 8-bit images and *Enhance Contrast* (under *Process*) by 3% in Image J. Then PLA puncta were quantified using the *Spot Detector* function in *Detection& Tracking* with the parameters for dual PLA (Detector= scale 1: 334 ; scale 2 : 60 ; scale 3 : 100 ; scale 4 : 1000; Filtering= Min size : 5 ; Max size : 25) or the parameters for single PLA (detector = scale 1 : 1000 / scale 2 : 89 / scale 3 : 1000 / Scale 4 : 1000 ; Filtering= Min size : 5 ; Max size : 25). These parameters were chosen manually on sample images in order to obtain optimal signal-to-noise ratio and then applied to all images for quantification. The names of functions used for image analysis are indicated in italics.

Quantification with BOPSS

1. *Color- and size-based segmentation of brightfield images into putative nuclei and non-nuclei*

Our custom BOPSS package first transforms RGB image pixels from PLA-brightfield images into the LAB color space (CIE International Commission on Illumination 1976). Unlike RGB, which specifies a red, green and blue value for each pixel, the LAB color space uses lightness, as well as green-red and blue-yellow opponency. The transformation between the red-green-blue (RGB) and LAB was accomplished using MATLAB’s built-in *makecform* function. The lightness channel spanned intensities from black (zero) to white (max). Typically, foreground pixels were lighter and had higher values in the lightness channel. Following transformation, a threshold was chosen automatically for each image using MATLAB’s built-in *graythresh* function. Briefly, *graythresh* computes an intensity value which splits the image pixels into two clusters. These two groups are produced to guarantee minimum intraclass variance values as compared to all other possible groupings. The threshold was applied to the lightness channel to select clusters of foreground pixels based on intensity and discard background pixels. The clusters of foreground pixels were then segregated into two groups (nuclei and putative puncta) based on their values in the A and B channels of the LAB color space. The group that had the larger average size for a cluster was labeled as the nuclei group and these clusters were excluded from further analysis. Within the cluster that was not excluded, a thresholding based on size is imposed: any cluster of pixels is discarded if its size is above the 95th percentile of all clusters from all images. This thresholding of putative puncta based solely on cluster size will tend to produce an upper limit on the number of real puncta as it categorizes any appropriately sized cluster as a punctum. Such clusters generally appear even in negative control images, typically around nuclei. The five percent of clusters with the largest sizes are categorized as nuclei (though they could also be other aggregates) and excluded from further analysis. Undercounting of puncta could occur for images completely lacking nuclei, though the number of puncta excluded from further analysis would be small. Two biological assumptions underlie the performance of this step: first, the majority of puncta should be located outside of nuclei, and second, puncta sizes are far smaller than nuclei. Because there should be virtually no D2R-A2AR dual PLA signal in nuclear areas, we safely exclude the largest pixel clusters in our analyses using BOPSS. By adjusting an internal parameter “Pct\_Cutoff”, the user can permit all clusters to pass this first step, regardless of size (this should only be done for images with very few large aggregates of pixels or nuclei). This first step was a highly permissive filter and passed most clusters of pixels that were smaller than nuclei. We referred to these passed clusters as putative puncta. However, because these putative puncta are present in both PLA treated and negative control images, a second step is necessary to learn the features of puncta present in images from PLA-treated samples.

1. *Naïve Bayesian classifier to categorize PLA puncta*

After discarding background and nuclear pixels in step 1, BOPSS generates distributions of pixel intensity and shape properties for each of the putative puncta. To do this, the program will first label each putative punctum based on its source image (treated image or a negative control image). BOPSS produces pairs of distributions (treated as well as negative control) over several features of each individual punctum. These features can be broadly described as shape/location features and intensity-based features. The shape/location features calculated for each punctum include: convex area, eccentricity, major axis length, minor axis length, orientation (angle), distance to the nearest neighboring cluster, and density of neighbors. The following intensity-based features are calculated for each punctum: mean pixel value, standard deviation of pixels, entropy of pixels, and range of pixels (highest valued minus lowest valued). Only 50% of the data (training data) is used to generate distributions characterizing the treated and the negative control puncta. In the next step, every remaining punctum (test data) is then classified based on its distance from all of the feature distributions. Each punctum is then classified as either signal or noise, depending on whether its features are, on the whole, closer to the center of the distributions for the puncta extracted from treated images (resulting in true puncta classification) versus puncta extracted from negative control images (resulting in noise classification). Thus, a punctum that has similarity to the features learned from the puncta used to train the algorithm will be classified as a PLA signal.

For the purposes of comparison, we consider “non-optimized” BOPSS (BOPSS\_0) puncta to be all puncta that passed the first step of BOPSS (color- and size-based segmentation), while “optimized” BOPSS puncta are those that passed both the first and second step (categorization by a naïve Bayesian classifier) (see Results).

*Statistical analysis*

Results were analyzed with Graphpad Prism (v8) and presented as mean ± the standard error of the mean (SEM). In the case of a fraction, the propagated SEM was calculated as:

(σ: SEM, z: the fraction; y and x: the mean of dual PLA and single PLA) [[6](#_ENREF_6)]. One-way ANOVA or Two-way ANOVA with post multiple comparisons test were performed to compare quantification results, as indicated in the figure legends.

**Supplemental Table 1. Human Subject information**

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Accession number | Days in PB buffer | SEX | Age | Cause of death | Postmortem Interval (hours from death to autopsy) | Blood Alcohol | Psychiatric Diagnosis | Comment | Brain Toxicology at CU | Neuropathology |
| PI12260 | 1951 | F | 68 | Myocardial infarction, acute. | 13 | 0 | Major Depressive Disorder. Single Episode Chronic, Melancholic, with Moderate Severity | No history of medication for depression | Cotinine | Encephalomalacia, subacute, small, in left ventrolateral thalamus. Atherosclerosis, moderate. |
| PI12270 | 920 | M | 47 | Myocardial infarction, acute. | 19 | 0 | NONE |  | Cotinine | Hypertensive vasculopathy |
| PI12277 | 921 | M | 50 | Hemorrhage from internal and external injuries. Pedestrian struck by car. | 21 | 0 | None | No frozen tissue | Not available | Small, acute hemorrhage in frontal horn of left lateral ventricle |

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**Supplemental Table 2. ROI, sampling areas and counting areas**

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Sample# | Full view image scale pixels/mm a | ROI (NAcc) area (mm2) b | Single sampling area (mm2) b | Single counting locus (mm2) b | Counting/ sampling (%) c | Number of Counting images d | | | Total Counting area /ROI (%) e | | |
| Dual | A2A | D2R | Dual | A2A | D2R |
| PI12260 | 40.02 | 36.32 | 2.72 | 0.11 | 4.04 | 13 | 6 | 11 | 3.94 | 1.82 | 3.33 |
| PI12270 | 41.96 | 84.9 | 1.68 | 0.11 | 6.55 | 25 | 31 | 27 | 3.24 | 4.02 | 3.50 |
| PI12277 | 39.91 | 47.7 | 2.75 | 0.11 | 4.00 | 34\* | 15 | 13 | 3.92 | 3.46 | 3.00 |

a.The values were derived from the scale of images produced by SCN400 viewer (Leica), the whole slide image processing software.

b. The size was measured with ImageJ with the scale of each sample (or image).

c. The percentage of one sampling area covered by the total of the counting loci.

d. The number of selected counting images for NAcc per PLA (suppl. Methods). One section per PLA condition was used, except for those marked with \* for which two sections were used.

e. The fractions were derived from total area of all counting images and the area of their corresponding ROIs

**Supplemental Figure Legends**

**Supplemental Figure. 1.** Sampling procedures for PLA-BF. Luxol fast blue/cresyl violet staining was performed to discriminate between white matter and grey matter (A-C). An outline of ventral striatum sub-territories (D) was drawn based on the stained results of each sample. Overlapping the sampling grid (E) and the outline (D) divided the brain section into several evenly distributed sampling areas (F and G). Within the NAcc, the ROI in this study (G), one counting locus (indicated by \* bounded by purple frame in the inset) was selected in each sampling area (indicated by the blue frame in the inset) and the 40x image of this counting locus was exported for quantification (H). A section of (H) is shown at high magnification (I). Scale bar, A-C, 5 mm; H-I, 50 µm.

**Supplemental Figure. 2.** Quantification of PLA signal with BOPSS and manual counting. Three randomly selected areas in a full counting image of each PLA condition, single (A), dual (B) and negative PLA (C) (from PI12277) were quantified with BOPSS or manually (four times independently). The puncta counted by BOPSS were marked in red in the representative images of pre-optimization (BOPSS\_0, D-F) and post-optimization analysis (BOPSS, G-I). The blue arrows indicated examples of reduced non-specific detection in post-optimization analysis. The manually counted puncta were marked in black and labelled with yellow numbers with Cell Counter (Image J) (J-L). The orange and white arrows indicate examples of overcounted and undercounted puncta detected by BOPSS compared with manual counting, respectively. One-way ANOVA was performed to analyze the results of single PLA. There is no significant difference among three quantification methods (P value=0.113) (M). Two-way ANOVA was performed to compare the quantification results for dual PLA and its negative control, which had the same PLA condition as dual PLA but omit one primary antibody (N). The interaction accounts for approximately 2.5 % of the total variance and is considered not significant (P value=0.069). Both quantification methods (accounts 33.9 % of the total variance, P value is <0.001) and PLA conditions (accounts 41.6 % of the total variance, P value is <0.0001) have significant effect on the variation. Bonferroni’s multiple comparison were performed to compare BOPSS and other methods (M and N), \*\*\*\* multiplicity adjusted P value <0.0001, \*\* <0.01.

**Supplemental Figure. 3.** Quantifying the signal of single PLA for A2AR and D2R, and dual PLA for D2R-A2AR, in the NAcc. The numbers of PLA puncta / mm2 were quantified by BOPSS (A-D, data were plotted as mean ± SEM). The fractions of D2R-A2AR dual PLA puncta relative to D2R (E) and A2AR (F) single PLA were calculated with the means in (A-C) and data were plotted as mean ± propagated error. The error was propagated as described in supplemental methods.

**References:**

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