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DetectSyn: A rapid, unbiased fluorescent method to detect changes in synapse density

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TITLE:

DetectSyn: A Rapid, Unbiased Fluorescent Method to Detect Changes in Synapse Density

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SUMMARY:

DetectSyn is an unbiased, rapid fluorescent assay that measures changes in relative synapse (pre- and postsynaptic engagement) number across treatments or disease states. This technique utilizes a proximity ligation technique that can be used both in cultured neurons and fixed tissue.

ABSTRACT:

Synapses are the site of communication between neurons. Neuronal circuit strength is related to synaptic density, and the breakdown of synapses is characteristic of disease states like major depressive disorder (MDD) and Alzheimer's disease. Traditional techniques to investigate synapse numbers include genetic expression of fluorescent markers (e.g., green fluorescent protein (GFP)), dyes that fill a neuron (e.g., carbocyanine dye, DiI), and immunofluorescent detection of spine markers (e.g., postsynaptic density 95 (PSD95)). A major caveat to these proxy techniques is that they only identify postsynaptic changes. Yet, a synapse is a connection between a presynaptic terminal and a postsynaptic spine. The gold standard for measuring synapse formation/elimination requires time-consuming electron microscopy or array tomography techniques. These techniques require specialized training and costly equipment. Further, only a limited number of neurons can be assessed and are used to represent changes to an entire brain region. DetectSyn is a rapid fluorescent technique that identifies changes to synapse formation or elimination due to a disease state or drug activity. DetectSyn utilizes a rapid proximity ligation assay to detect juxtaposed pre- and postsynaptic proteins and standard fluorescent microscopy, a technique readily available to most laboratories. Fluorescent detection of the resulting puncta allows for quick and unbiased analysis of experiments. DetectSyn provides more representative results than electron microscopy because larger areas can be analyzed than a limited number of fluorescent neurons. Moreover, DetectSyn works for *in vitro* cultured neurons and fixed tissue slices. Finally, a method is provided to analyze the data collected from

this technique. Overall, DetectSyn offers a procedure for detecting relative changes in synapse density across treatments or disease states and is more accessible than traditional techniques.

INTRODUCTION:

Synapses are the fundamental unit of communication between neurons[1]. Many synapses between neurons within the same regions give rise to circuits that mediate behavior[2]. Synapses consist of a presynaptic terminal from one neuron that releases neurotransmitters or neuropeptides that relay information to postsynaptic receptors of another neuron. The summation of presynaptic signals determines whether the postsynaptic neuron will fire an action potential and propagate the message to other neurons.

Synaptopathology, the break down of synapses, arises in diseases and disorders marked by decreased neural volume, like Alzheimer's disease and major depressive disorder, resulting in circuits that no longer optimally perform[3–5]. Restoring synapse density likely underlies the efficacy of potential treatments for these disorders. For example, it was recently demonstrated that increasing synapses underlies the behavioral efficacy of rapid antidepressants[6]. To rapidly screen possible synaptopathology treatments, researchers require techniques that quickly identify changes to synapse numbers.

Current methodologies are either time-consuming and expensive (electron microscopy, array tomography), or they only examine postsynaptic changes without incorporating presynaptic engagement (spine analyses, immunofluorescence/colocalization). Dyes like DiI or fluorescent proteins like GFP help visualize neurons and characterize postsynaptic spines. However, spine analysis uses researcher-defined ratios to determine morphology, which can decrease reproducibility[7]. Further, how the different spine classes relate to functional synapses is still being uncovered[8]. Spine formation can be transient and may reflect postsynaptic plasticity, but these spines could be eliminated before stabilizing into a synapse with a presynaptic neuron[9].

Colocalization provides a better proxy for synapses than spine analysis because one can immunostain for presynaptic and postsynaptic proteins. However, synaptic proteins may yield low colocalization values because the proteins are juxtaposed and may not consistently overlap. Thus, because the proteins are not wholly superimposed, colocalization techniques may not accurately measure changes to synapse formation due to this missing information. Finally, although both electron microscopy (EM) and array tomography provide high-resolution images of synapses, they are time-consuming. EM further requires specialized equipment, and researchers are limited to small volumes of tissue for any given experiment. While array tomography elegantly provides the ability to screen for many proteins on ultrathin sections and can be combined with EM[10], this technique may be too labor-intensive and beyond the scope of experiments that need to scan rapidly for changes to synapse formation.

DetectSyn is a specific application of the Duolink Proximity Ligation Assay. The PLA assay allows for the general detection of protein-protein interactions. DetectSyn bridges proxy postsynaptic measures by amplifying a fluorescent signal emitted by tagged pre- and postsynaptic proteins within 40 nm of each other. If the synaptic proteins are within 40 nm, as within a synaptic cleft,

then the secondary antibodies, which contain DNA probes, will hybridize into circular DNA. This hybridized circular DNA expresses a fluorescent probe, which is then amplified and detected with standard fluorescent microscopy techniques (see **Figure 1**). Crucially, unlike EM and array tomography, this technique does not require specialized equipment and takes about the same amount of time as standard immunohistochemistry. The accessibility of this technique, thus, enables investigators outside of research-intensive institutions to participate in synaptopathology research. Further, this technique can examine changes to synaptic density in multiple brain regions within a single experiment, offering a more holistic representation of synaptic changes due to disease or treatment.

PROTOCOL:

Isolation of cells and tissue from animals was in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and approved by the Wake Forest Institutional Animal Care and Use Committee

NOTE: This protocol is used on samples already treated and fixed per specific experimental paradigms and requirements. For demonstration purposes, synapse formation due to rapid antidepressant treatment is used to highlight this synapse detection technique[6]. Neurons previously cultured on coverslips, treated, fixed in 4% paraformaldehyde (PFA), and stored in 1x phosphate-buffered saline (PBS) will be used to highlight the *in vitro* procedures. Previously sliced hippocampal tissue (25 µm thick) from mice treated, transcardially perfused with ice-cold PBS and 4% PFA, and then stored in cryoprotectant will be used to highlight the slice procedures. Please see[11,12] for more information about how to culture neurons or transcardially perfuse rodents. See **Figure 1** for a graphical representation of this procedure.

[Place **Figure 1** here.]

1. Rinse samples

1.1. Rinse the samples with 500 µL of 1x PBS + 0.75% glycine for 5 min 3 times with gentle agitation on an orbital shaker to remove residual PFA or cryoprotectant.

2. Block and permeabilize samples

2.1. Prepare blocking and permeabilization solution (10% normal donkey serum, 0.25% Tween 20) in 1x PBS. Prepare enough to use for blocking, primary, and secondary incubations.

2.2. To samples (e.g., coverslips or free-floating slices) in 24 well plates, add 500 µL of blocking and permeabilization solution. Ensure each well contains a different sample and is appropriately labeled to prevent samples from being switched.

2.3. Incubate the samples at room temperature (RT) for 60 min for cultured cells or 2 h for sliced tissue. Use an orbital shaker for gentle agitation.

3. Incubate samples in primary antibodies

3.1. Prepare primary antibodies in blocking buffer:

3.1.1. Prepare Postsynaptic density 95 (PSD95; 1:500, rabbit polyclonal), Synapsin1 (1:500, mouse monoclonal), MAP2 (1:400, chicken polyclonal)

3.1.2. Prepare a negative control aliquot that omits one of the synaptic pairs (e.g., without PSD95)

3.2. Carefully remove the blocking solution with a plastic Pasteur pipette. Try to remove as much as possible without disturbing the cells or tearing tissue.

3.3. For cultured cells:

3.3.1. Line a large plastic petri dish with parafilm. Carefully transfer coverslips to the parafilm using forceps.

3.3.2. Carefully add 60 µL of the primary antibody solution to the top of the coverslips. Make sure not to spill the primary antibody solution over the side of the coverslip.

3.3.3. To provide humidity and prevent samples from drying out during the incubation period, add ultrapure water to a smaller Petri dish and carefully arrange the small Petri dish around the coverslips.

3.3.4. Cover the large Petri dish and incubate cultured cells for 1 h at RT.

3.4. For sliced tissue:

3.4.1. Carefully add 250 µL of the primary antibody solution to free-floating slices in a 24 well plate.

3.4.2. Cover the plate and incubate the tissue overnight at 4 °C with gentle agitation on an orbital shaker.

4. Wash samples, then incubate in secondary antibodies

4.1. Prepare secondary antibodies in the blocking buffer:

4.1.1. Prepare Donkey anti-mouse (1:5), donkey anti-rabbit (1:5), donkey anti-chicken (1:400).

4.1.2. At this step, additional technical controls can be obtained by preparing a secondary aliquot that omits either the anti-mouse or anti-rabbit secondary.

4.2. For cultured cells:

4.2.1. Using forceps, carefully tap off the primary solution from coverslips onto a paper towel

4.2.2. Using forceps, carefully transfer the coverslips back to their original 24 well plate filled with 500 µL of 1x PBS.

4.3. For sliced tissue:

4.3.1. Carefully remove the primary antibody solution with a plastic Pasteur pipette. Try to remove as much as possible without tearing tissue.

4.3.2. Add 500 µL of 1x PBS

4.4. Wash the samples for 10 min 3 times in 1x PBS with gentle agitation on an orbital shaker. During this time, bring all wash buffers to RT.

4.4.1. During this time, change the parafilm in the large Petri dish

4.5. For cultured cells:

4.5.1. Using forceps, carefully transfer the coverslips back to the parafilmed large Petri dish

4.5.2. Carefully add 40 µL of the secondary antibody solution to the top of the coverslips. Make sure not to spill the secondary antibody solution over the side of the coverslip.

4.5.3. If needed, add more ultrapure water to a smaller petri dish and carefully arrange the small petri dish around coverslips.

4.5.4. Cover the large Petri dish

4.6. For sliced tissue:

4.6.1. Carefully add 250 µL of the secondary antibody solution to free-floating slices in a 24 well plate.

4.6.2. Cover the plate

NOTE: From here on, protect samples from light by wrapping the tops of the plates with foil.

4.7. Incubate the samples at 37 °C for 1 h

5. Ligation

5.1. Mix the ligation stock 1:5 in molecular-grade water.

5.2. As in section 4, carefully transfer the coverslips and remove the secondary mix from the sliced tissue.

5.3. Wash the samples in 500 µL of wash buffer A

5.3.1. For cultured cells, wash 2 times for 5 min. For sliced tissue, wash 2 times for 10 min. Use gentle agitation on an orbital shaker for both.

5.3.2. During this time, change the parafilm in the large Petri dish

5.4. While keeping the ligase on a cold block, dilute the ligase 1:40 in the ligation stock from step 5.1. Perform this dilution immediately before adding the ligase to the samples.

5.5. As in section 4, remove as much of the wash buffer A as possible from samples before adding the ligase.

5.6. For cultured cells: Transfer coverslips back to the parafilmed Petri dish. Add 40 µL of the ligation mix to coverslips, arrange small water-filled Petri dishes around the coverslips, and cover the large Petri dish.

5.7. For sliced tissue: Add 250 µL of the ligation mix from Step 5.4 to each well and cover the plate.

5.8. Incubate the samples for 30 min at 37 °C.

6. Amplification

6.1. Mix the amplification stock 1:5 in molecular-grade water.

6.2. As in section 4, carefully transfer the coverslips and remove the ligation mix from the sliced tissue.

6.3. Wash samples in 500 µL of wash buffer A

6.3.1. For cultured cells, wash 2 times for 2 min. For sliced tissue, wash 2 times for 10 min. Use gentle agitation on an orbital shaker for both.

6.3.2. During this time, change the parafilm in the large Petri dish

6.4. Perform this dilution immediately before adding the polymerase to samples. While keeping the polymerase on a cold block, dilute polymerase

6.4.1. For cultured cells, dilute polymerase 1:80 in the amplification stock from step 6.1.

6.4.2. For sliced tissue, dilute polymerase 1:40 in the amplification stock from step 6.1.

6.5. As in step 4, remove as much of the wash buffer A as possible from samples before adding the polymerase.

6.6. For cultured cells: Transfer the coverslips back to the parafilmed Petri dish. Add 40 µL of the amplification mix from Step 6.4.1 to coverslips, arrange small water-filled Petri dishes around the coverslips, and cover the large Petri dish. Incubate the samples for 100 min at 37 °C.

6.7. For sliced tissue: Add 250 µL of the amplification mix from Step 6.4.2 to each well and cover the plate. Incubate the samples for 2 h at 37 °C.

NOTE: During this time, prepare and label slides.

7. Mounting

7.1. As in Step 4, carefully transfer the coverslips and remove the amplification mix from the sliced tissue.

7.2. Wash the samples in 500 µL of wash buffer B 2 times for 10 min with gentle agitation on an orbital shaker.

7.3. Wash the samples in 500 µL of 1% wash buffer B for 1 min with gentle agitation on an orbital shaker.

7.4. For cultured cells:

7.4.1. Drop 3 µL of mounting media onto a slide

7.4.2. Tap off excess wash buffer from the coverslip and then place the coverslip (with cells facing down) into the mounting media. Seal the sides with a small amount of clear nail polish to seal the coverslip in place.

7.5. For sliced tissue:

7.5.1. Carefully transfer a tissue slice to the prepared slide and arrange it, so the slice is lying flat. Drop between 5–10 µL of mounting media (amount will depend on the size of the slice) onto tissue slice

7.5.2. Carefully place a glass coverslip over tissue slice and seal with a small amount of clear nail polish along the edge to seal the coverslip in place.

7.6. Wait at least 15 min before analyzing under the microscope, or store at -20 °C.

8. Obtain digital images with a confocal microscope.

8.1.1. Optimize the acquisition settings (e.g., laser power, gain, offset) across samples from all treatments. Ensure that the optimization includes decreasing background noise and enhancing the signal without oversaturating the intensity of the fluorescent signals. Once settings are determined, apply the same acquisition settings across all images obtained.

NOTE: The following acquisition details can be used with a Nikon A1 confocal microscope and the Nikon NIS AR Elements software.

8.1.2. Set the slide with sample on the stage and find the focal plane for the sample using DAPI through the eyepiece.

8.1.3. Turn off the eye port by clicking on **Eye port** and choose an optical configuration button to adjust the settings.

8.1.4. Adjust the gain, offset, and laser power for each fluorescent channel to decrease background noise and enhance the fluorescent signal. Make sure the fluorescent signal does not become oversaturated as mentioned in steps 8.1.5–8.1.6.

8.1.5. Monitor oversaturation using a pseudocolor for the fluorescent signal. At the bottom of the live image, right-click the tab labeled with the fluorescent channel currently being used.

8.1.6. Next, choose **Channel Coloring** and pick a pseudocolor like **Rainbow Dark** to visualize the fluorescence intensity in a heat-map-like pseudocolor. In Rainbow Dark, cooler colors indicate less fluorescent intensity, and hotter colors indicate more fluorescent intensity.

8.1.7. Once all fluorescent channels are optimized, right-click on the optical configuration button previously chosen and choose **Assign Current Camera Setting** for this button.

8.1.8. Verify that the chosen settings are sufficient for a random sample from each treatment group. If the chosen settings oversaturate any of these samples, repeat step 8.1.4 to eliminate the oversaturation.

8.1.9. For cultured neurons, follow steps 8.1.10–8.1.16.

8.1.10. Using the eye-port, search for a neuron with dendrites that have minimal overlap with other dendrites.

8.1.11. Turn off the eye port and use the DAPI channel to visualize the cell body of the chosen neuron. Double-click on the center of the soma to center the neuron in the middle of the field of

view.

8.1.12. Using the MAP2 channel, find the best plane of focus for the MAP2 signal with live scanning.

8.1.13. Under the **ND Acquisition** tab, click on **Save to File** and choose a file to save the image into under **Browse**. Then, input the filename.

8.1.14. Under the **Z** tab, select the **Symmetric Mode Defined by Range** option. Set the focus to the best MAP2 plane and click on the **Relative button** to set this focal plane as the middle of the z-stack.

8.1.15. Set the range to 5 µm with 1 µm steps, and make sure to check **Close Active Shutter During Z Movement**. Under the **Wavelength** tab, select the name of the optical button with the previously configured acquisition settings under **Optical Conf**. Then, click on **Run Now**.

8.1.16. Repeat steps 8.1.10–8.1.15for about 10 neurons per coverslip/treatment.

8.1.17. For sliced tissue, follow steps 8.1.18–8.1.22

8.1.18. Using the eye-port, search for the region of interest. For example, locate CA1 of the hippocampus.

8.1.19. Turn off the eye-port and use the MAP2 channel to find the best plane of focus for the MAP2 signal with live scanning.

8.1.20. Under the **Acquire** menu, choose **Scan Large Image**. Next, select the optical button's name with the previously configured acquisition settings under the **Capturing** panel from the panel that opens. Also, ensure to select the correct objective in this panel.

8.1.21. Under the **Area** panel and the eyepiece, use the arrow keys to set the boundaries of the region of interest. Next, click on **Save Large Image to File** and create a save path filename for the image.

8.1.22. Under the **Setup** panel, make sure **Multichannel Capture** is checked, and then choose the name of the optical button with the previously configured acquisition settings under **Optical Conf**.

NOTE: A z-stack for a large image is possible but will increase the scan time.

9. Analysis

9.1.1. Similar to acquisition settings, use samples from all treatments to optimize threshold settings. Ensure that the threshold optimization focuses on decreasing background noise and

enhancing the signal without oversaturating the intensity of the fluorescent signals. Once these settings are determined, apply the same threshold settings across all images used for analysis as described in steps 9.1.2–9.1.3.

9.1.2. In ImageJ, the threshold option is located under the menu **Image** > **Adjust** > **Threshold**. Choose the **Dark Background** option if the image has a dark background.

9.1.3. Next, adjust the upper and lower bounds of the threshold per previously determined optimized threshold settings, then click on **Apply**.

9.1.4. For cultured cells, use the MAP2 channel and a freehand region of interest (ROI) tool to draw an ROI for each neuron, including dendrites and soma. For sliced tissue, draw a freehand ROI within the slice image that encapsulates the area of interest (e.g., stratum radiatum of the CA1 within the hippocampus).

9.1.5. Obtain the area of the ROI. In ImageJ, measure the area under the menu **Analyze** > **Measure**.

9.1.6. Detect the number of puncta within each ROI using an automatic detection tool like Particle Analysis in ImageJ following steps 9.1.7–9.1.9.

9.1.7. Find the Particle Analysis option under the menu **Analyze** > **Analyze Particles**. First, define the puncta size diameter, typically 0.1–3 μm^2.

9.1.8. Next, choose the **Overlay Masks** option from the **Show** drop-down menu and check the **Display Results** option. Then, click on **OK**.

9.1.9. If puncta are not detected with the diameter range chosen, adjust the range until all puncta are detected with this analysis. Make sure to use the same Particle Analysis settings for all images.

9.1.10. Divide the number of puncta by the area of an individual region of interest by following steps 9.1.11–9.1.13.

9.1.11. Copy and paste the results for each image from the **Results** pop-up from ImageJ into a spreadsheet.

9.1.12. First, identify which file and sample the data were obtained from. Then, divide the area of the ROI by the number of puncta.

9.1.13. Then, clear the data from the **Results** pop-up, and repeat steps 9.1.2–9.1.12.

9.1.14. Normalize results to control samples: Average the results (number of puncta/ROI area) for the control samples. Then, divide the obtained results of all samples by the average of the

control to obtain the normalized results. The new average of the control samples should be equal to 1.

REPRESENTATIVE RESULTS:

Data modified from Heaney et al.[6] are presented to demonstrate an experiment where increased synapse formation is expected (please see [6] for more information and a more in-depth discussion of the mechanism). Previously, it was demonstrated that rapid antidepressants require activation of the inhibitory metabotropic receptor, GABAB (gamma-aminobutyric acid subtype B), to be effective[13]. Further, previous data indicated that rapid antidepressants increase postsynaptic markers[14]; thus, DetectSyn was used to test the hypothesis that rapid antidepressants increase synapse numbers to be effective.

In cultured neurons, four conditions are tested. First, in the top panel of **Figure 2A**, cultured neurons were treated with the vehicle under the same conditions as the experimental control. However, the PSD95 primary antibody is omitted to provide a technical control for the DetectSyn assay (see **Figure 1** for how each component contributes to the signal). Next, in the Control panel, neurons again are treated with the vehicle but receive both PSD95 and Synapsin1 primaries. Next, cultured neurons are treated only with the rapid antidepressant Ro-25-6981 (Ro) or Ro plus the GABAB agonist, baclofen (Bac). As previously demonstrated[13], both the rapid antidepressant Ro-25-6981 and the GABAB agonist baclofen are required for the *in vitro* efficacy of Ro-25-6981. Thus, an increase in synapse formation is demonstrated by an increase in white puncta (**Figure 2A**). Without baclofen, no increase in synapse formation *in vitro* is seen.

In vivo, basal GABA levels are sufficient for the rapid antidepressant to work[13], so only the rapid antidepressant Ro-25-6981 is administered via intraperitoneal injection to mice (**Figure 2B**). The left panel of Fig. 2B represents another technical control for the DetectSyn assay. Hippocampal tissue from a saline-treated mouse was probed with both primaries for PSD95 and Synapsin1, but one of the secondaries was omitted. An increase in synapse formation due to treatment with the rapid antidepressant Ro-25-6981 compared to saline-treated mice is demonstrated in the middle and right panels of **Figure 2B**.

Representative images for technical controls *in vitro* and *ex vivo* are shown. In **Figure 2A**, one of the primaries for the synaptic pairs is omitted (i.e., PSD95), and in **Figure 2B**, one of the secondaries is omitted. While some puncta appear in the technical control images, they are generally not the same size, as demonstrated by the white speck in the top panel of **Figure 2A** compared to the large puncta in the other panels. Further, these puncta are not in the same location as the puncta quantified, as demonstrated by some puncta appearing within the soma of the technical control in **Figure 2B**. Typically, non-specific puncta occur within the nuclei, perhaps due to the presence of DNA.

To analyze the representative results in **Figure 2A**, dendrites (visualized by MAP2 staining and represented here in a grey outline) were traced using a freehand drawing tool to create regions of interest (ROIs). First, the area of the MAP2 ROIs was obtained using the NIS Elements function, ROI Data under the **Automated Measurement Results** tab. Next, puncta were detected using the

Thresholding function in NIS Elements. This function creates a binary mask of objects that fall within a defined intensity threshold. Then, the number of binary objects within the MAP2 ROIs were detected using the NIS Elements function, Binary in ROI, under the **Automated Measurement Results** tab. The data presented in the graph to the right of **Figure 2A** are the normalized values of the puncta divided by the area of the ROI.

To analyze the representative results in **Figure 2B**, the stratum radiatum of the CA1 was traced using a freehand drawing tool to create an ROI. First, the area of the ROI was obtained using the ROI data function, as described in the paragraph above. Next, puncta were detected using the **Spot Detection – Bright Spots** function, located under the **Binary** menu. Next, the diameter and contrast values were chosen based on visual inspection of multiple slices from all treatments. Once these values were decided, they were applied uniformly across all analyzed images. The **Spot Detection** function creates binary masks of objects within the defined diameter and contrast parameters set. Then, the number of binary objects within the MAP2 ROIs were detected using the NIS Elements function, Binary in ROI, under the **Automated Measurement Results** tab. The data presented in the graph to the right of **Figure 2B** are the normalized values of the puncta divided by the area of the ROI.

FIGURE AND TABLE LEGENDS:

Figure 1: Graphical representation of DetectSyn assay. After permeabilizing cell membranes, primary antibodies for Synapsin1 and PSD95 bind to these synaptic proteins. Secondaries with oligonucleotide tags then bind to the primary antibodies. If Synapsin1 and PSD95 are within 40 nm, as at a synapse, then the oligonucleotides interact, and a fluorescent tag is amplified. This fluorescent signal can then be imaged via standard microscopy and analyzed.

Figure 2: Detection of synapses using DetectSyn assay. White puncta represent synapses detected with DetectSyn PSD95-Synapsin1 proximity ligation assay (yellow arrowheads) in (**A**) *in vitro* cultured primary hippocampal neurons and (**B**) *ex vivo* CA1 stratum radiatum. In (**A**), the grey outline represents MAP2 staining. In (**A**), the top panel labeled PLA control represents samples treated with only vehicle, like the Control. However, the PSD95 primary was not included in the reaction. In the final two panels, neurons were treated with the rapid antidepressant Ro-25-6981 (Ro, 10 µM) or Ro plus the GABAB agonist baclofen (Bac, 50 µM) for 90 min. The increased number of puncta (identified with yellow arrowheads) indicates that *in vitro*, synapse formation only increases when the rapid antidepressant Ro-25-6981 is administered with the GABAB agonist. In (**B**), green represents dendrites stained with MAP2, and blue represents nuclei stained with DAPI. Single dendrites, outlined in yellow rectangles in the merged images, are isolated beneath representative images to demonstrate that puncta localize to dendrites. Animals were treated with Ro-25-6981 (10 mg/kg), and tissue was collected 45 min after treatment. The increased number of puncta (identified by yellow arrowheads) indicates that *in vivo*, the number of synapses increases with basal GABA signaling and the rapid antidepressant Ro-25-6981. Quantification of representative results is represented by the bar graphs and indicates the average number of DetectSyn puncta divided by MAP2 area. Results are normalized to the experimental control. Images were obtained using a Nikon A1plus confocal microscope. Scale bars = (**A**) 10 µm; (**B**, top) 50 µm; (**B**, bottom) 5 µm. Bars represent the mean +/- standard

error of the mean. Results analyzed by one-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by post-hoc analysis. The figure has been modified from Heaney et al.[6].

DISCUSSION:

DetectSyn is a rapid assay that uses a proximity ligation assay to detect proteins within 40 nm of each other, which allows for the detection of synapse formation. This technique improves current fluorescent assays, which serve only as proxy measurements for synapse formation. DetectSyn detects quantifiable changes in synaptic proteins localized within 40 nm, i.e., within the synaptic cleft, of each other. Further, DetectSyn is more cost-effective and takes less time than techniques, like electron microscopy and array tomography, designed to measure synapses and classically designated as gold standard techniques.

DetectSyn is a highly customizable technique. Any synaptic pair can be used to identify the formation of specific types of synapses, as long as the protein pair reside within 40 nm of each other. While this exciting technique has many applications, one must take care to validate antibodies, including optimal blocking and primary incubation conditions, and continue to support their results using other methods like immunofluorescence. It is suggested to perform these supporting experiments in separate experiments that do not utilize DetectSyn to decrease antibody binding competition (e.g., using goat anti-PSD95 and mouse anti-PSD95 in the same experiment may reduce binding due to competition). Additionally, take care to choose primary antibodies raised in different hosts—for example, goat anti-PSD95 and rabbit anti-Synapsin1. If the same host is used for both primaries, the secondary probes will not discriminate between the two primaries.

Further, because proximity ligation has an amplification step, there is limited information one can draw from the resulting puncta. For instance, different sized puncta are observed (evident in Figure 2B), suggesting a change in synapse size or multiple synapses recruited to the same area. However, even though each sample within an experiment undergoes the same conditions, the amplification step makes it difficult to draw these conclusions categorically.

Finally, critical steps for this protocol include ensuring the ligase and polymerase remain on an ice block and are added as quickly as possible to samples. Additionally, including technical controls will help identify where non-specific puncta may appear in a given experiment. For example, as seen in Figure 2B, puncta within nuclei are seen in technical controls. Thus, it is crucial to include negative or technical controls to ensure that the signal detected in experimental samples is greater than that seen in these controls. Typically, these technical controls are obtained by omitting one of the primary or secondary pairs. Finally, ensure incubation temperatures are at 37 °C starting at the secondary step.

Importantly, DetectSyn provides an accessible way for almost any laboratory with access to a standard fluorescent microscope, regardless of technical experience, to study synaptopathologies.

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DISCLOSURES:

The authors report no conflict of interest.

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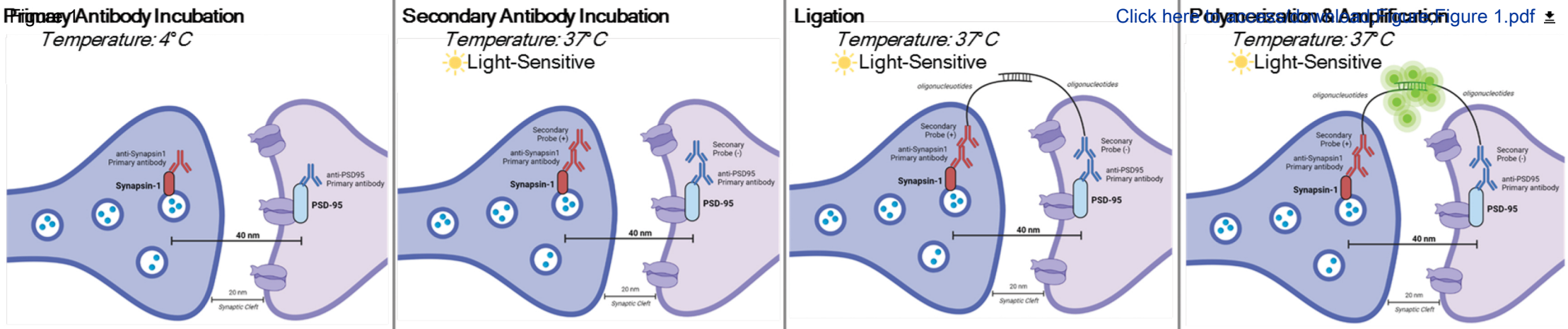
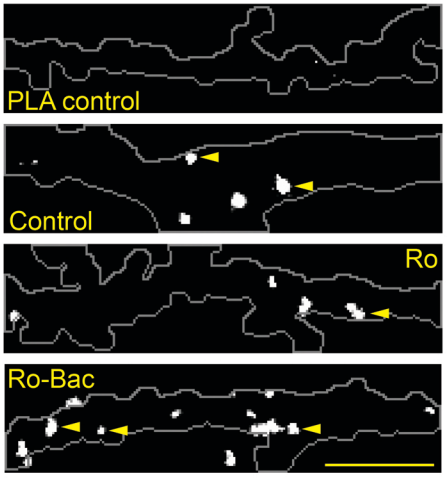
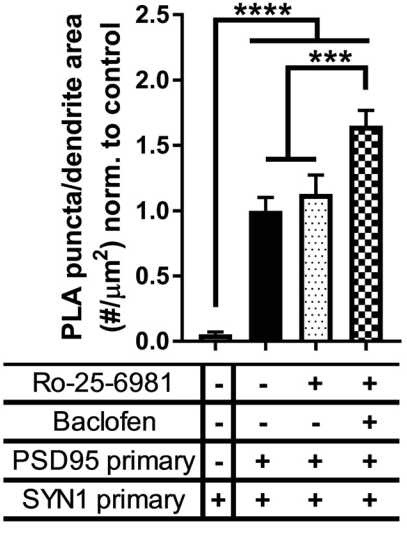


Figure 2

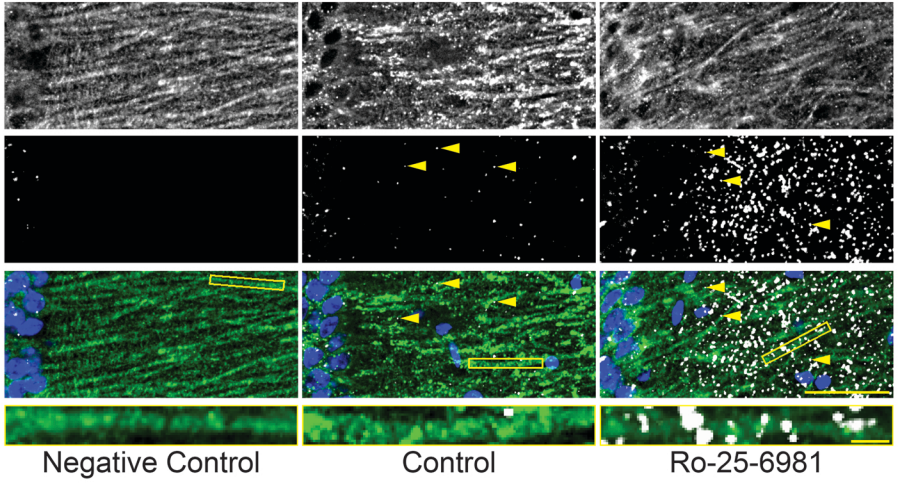
A.



MAP2 mask + PSD95/SYN1 PLA puncta



B.



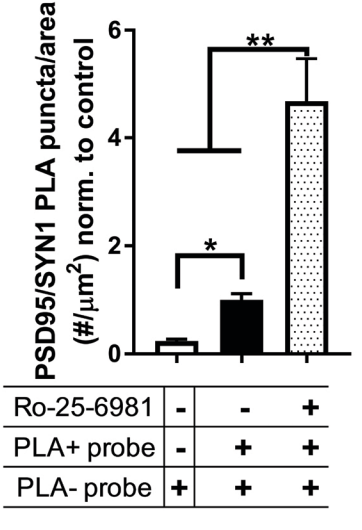


Table of Materials

We want to thank the Editor and Reviewers for their careful consideration of this manuscript. We feel that by addressing the Editor's and Reviewers' keen comments and concerns, we have significantly strengthened and clarified the manuscript. In what follows, italicized text corresponds to the Editor's and Reviewer's comments, and normal typeface corresponds to our replies and changes to the manuscript.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We thank the Editor for their comments. We have proofread the manuscript for spelling and grammatical issues.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have removed personal pronouns from the text.

3. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: Duolink, Nikon A1plus, etc.

As much as possible, we have removed commercial language.

4. Use SI units as much as possible and abbreviate all units: L, mL, µL, cm, kg, etc. Use h, min, s, for hour, minute, second.

We have verified the use of SI units throughout the manuscript.

5. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have removed personal pronouns from the text.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We thank the Editor for their comments. We have updated the steps to be more detailed.

7. Step 2.2: Were the coverslips and tissue slices placed in the same well or different wells?

We have updated Step 2.2 to reflect samples from individual treatments should be kept separate.

8. Step 3.3.4: What is the rationale for using the water filled Petri dish? Is it kept for humidification? Please describe in 1-2 lines.

We have updated Step 3.3.4 to reflect the purpose of this step.

9. Step 3.3.5: Do the coverslips have adhered cells on them? If yes, please specify this. If not, please mention at what step were the cells added.

Step 3.3.5 has been updated to be clearer. We also mention in the NOTE before the beginning of the protocol that coverslips have cells adhered.

10. Step 4.2.1: How was the primary solution tapped off?

We have updated Step 4.2.1. to reflect how the solution is tapped off.

11. Step 4.2.2, 4.3.2: What was the concentration of PBS+glycine used.

Steps 4.2.2 and 4.3.2 have been updated to include the concentration of PBS and glycine used.

12. Step 7.4.1, 7.5.2: What was the concentration of DAPI used?

For these steps, DAPI is included in the mounting media and we were unable to find the concentration on the manufacturer's website. These steps were also updated to remove commercial name of the mounting media.

13. Step 7.7: How were the slides set up for imaging? How were the images acquired? Please elaborate the steps for acquiring images. Please mention all the steps that are necessary to execute the action item. If this step needs to be filmed, please make sure to provide all the details such as "click this", "select that", "observe this", etc. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. Please keep in mind that software steps without a graphical user interface (GUI) cannot be filmed.

We have significantly elaborated on Step 7.7 to reflect how we obtain images.

14. Step 8: Did the regions of interest have a fixed shape or size or were they drawn using the free-hand tool? How were the puncta identified? Was it done manually? How were they counted? Manually or using the analysis system program? How was the area of region of interest calculated? How was the normalization done? Please provide all details necessary to perform the analysis. Alternatively, add references to published material specifying how to perform the protocol action. Readers of all levels of experience and expertise should be able to follow your protocol.

We have significantly elaborated on Step 8 to reflect the analysis process.

15. Please include a single line space between all the steps and sub-steps. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have updated the text to include a single line space between steps and sub-steps. We have highlighted the essential steps to be recorded.

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17. Please ensure that the Representative Results section explains the results in the context of the

technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

We have significantly updated the representative results section to include more information about how the representative results show the technique. We also confirmed the text refers to all of the figures.

18. Please ensure that all the essentials (chemicals, reagents, consumables, equipment, etc.) used in this study are included in the Table of Materials.

We have updated the Table of Materials to include all essential materials.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This is well written protocol manuscript that describes a procedure for performing synapse detection using proximity ligase assay to identify pre- and postsynaptic elements within 40 nm of each other. The authors present this as an excellent compromise between the "gold standard" electron microscopy or array tomography (which are expensive) and postsynaptic spine detection or staining for spine proteins (which can have a high false detection rate). The explanations are clear and procedure is described in sufficient detail to follow. I have only a few comments.

We thank Reviewer 1 for their support of our manuscript and procedures.

Major Concerns:

The procedure does require two steps of incubation at 37C. Not every lab has access to an incubator. Would a water bath be a possible alternative?

A water bath may not be the best alternative because of the instability of water, the risk of diluting reagents and disrupting/contaminating samples with the bath water. However, it is conceivable to create a humidity chamber using a water bath and clipping a reusable plastic container to the side of the water bath for stability. We have updated our Table of Materials to include an incubator.

Minor Concerns:

The authors make a one sentence comparison of their DetectSyn approach to standard immunohistochemistry double labeling pre- and postsynaptic proteins with colocalization (line 82-83). The authors should provide some additional details about why standard colocalization is not sufficient.

We thank the Reviewer for their comment. We have added more information about why standard colocalization is not sufficient from lines 79-82:

However, synaptic proteins may yield low colocalization values because the proteins are juxtaposed and may not consistently overlap. Thus, because the proteins are not wholly superimposed, colocalization techniques may not accurately measure changes to synapse formation due to this missing information.

What is the purpose of the MAP2 antibody?

Here, we use MAP2 to identify dendrites, our region of interest. This marker can be swapped out depending on varying experimental needs. We have updated our text to reflect the purpose of MAP2.

What is the purpose of 0.75% glycine n the initial rinse from fixative and/or cryoprotectant.

We use 0.75% glycine in the initial PBS rinse to remove trace formaldehyde from the samples, and to decrease background noise. We have updated our text to reflect the purpose of the glycine in the PBS wash.

Reviewer #2:
Manuscript Summary:
Review of Jove 63139

The work is of potential interest but is totally missing an important explanation of the technique. I had to look on the Sigma web site searching for DuoLink before I could figure out what they were talking about (also guided by their Fig. 1). In what way is their explanation different than what one gets from the Sigma web site?

We thank Reviewer 2 for their comments. We have updated the manuscript to include a more detailed protocol for analysis. Further, we provide information on how to use this application for synapse detection and how to use the application in slice tissue, which is not provided on the Sigma website. We also thank Reviewer 2 for their comments about Figure 1, which indicated this figure was not as helpful as we intended. Thus, we also updated our figure to explain the technique more clearly.

The reviewers need to have a paragraph or two discussing what DuoLink Proximity Ligation Assay is and what is unique about their application. (The web-site Olink.com has many neuroscience applications.) I presume they are using ligase and rolling circle amplification; how much amplification do they achieve?

We thank the Reviewer for their comments. We have updated our last introduction paragraph to include a discussion of the Duolink PLA and how our application is unique.

Figure 2 is their only data which they actually show, but I find the explanation awfully short. Figure 2A, left-hand-side, I don't really know what the figure means—the grey lines in the PLA control? How are the number of spots in the control vs Ro mean? Fig 2B. : what is the negative control vs Control?

We thank the Reviewer for their comments and concerns. We have significantly updated our legend and representative results to more thoroughly explain our results.

Major Concerns:
They don't bother to explain their technique! How is this different than what one can get from Sigma web link?

We have included more information about the technique and the benefits of using this application of the Duolink technique. Further, we provide a detailed explanation of how to analyze the results obtained using cultured neurons or sliced tissue, which is not available on the Sigma website.

The representative results are adapted from [1], which was published as an open access article. The following links and screenshots indicate the open access policy that allows for the adaptation and distribution of the material, as long as it is appropriately credited and cited. We have made sure to credit and cite the work throughout the JoVE article properly.

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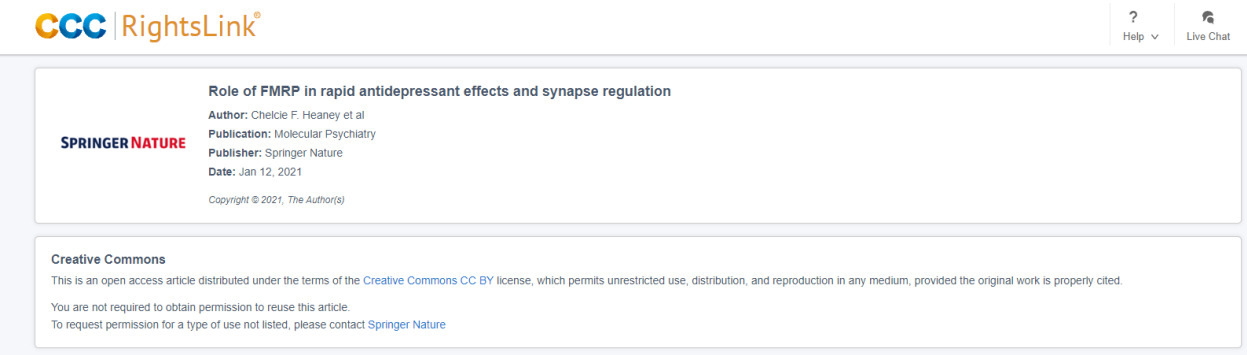

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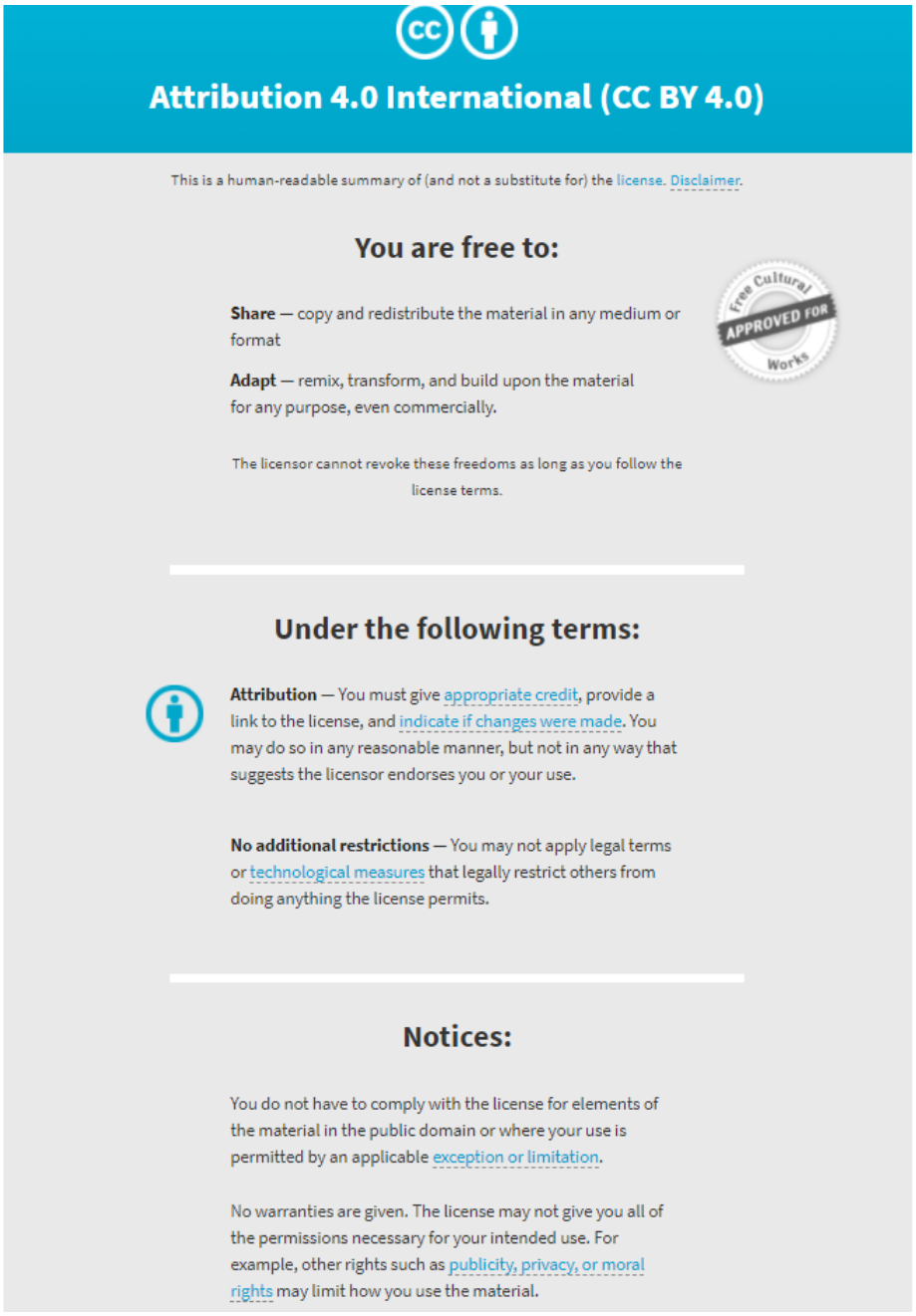

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1. Heaney, C.F., Namjoshi, S.V., Uneri, A., Bach, E.C., Weiner, J.L., Raab-Graham, K.F. Role of FMRP in rapid antidepressant effects and synapse regulation. *Molecular Psychiatry*. 26 (6), 2350–2362, doi: 10.1038/s41380-020-00977-z (2021).