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Corresponding Author:	Susana Restrepo University of Colorado Denver - Anschutz Medical Campus Aurora, Colorado UNITED STATES
Corresponding Author's Institution:	University of Colorado Denver - Anschutz Medical Campus
Corresponding Author E-Mail:	SUSANA.RESTREPO@cuanschutz.edu
Order of Authors:	Susana Restrepo
	Samantha L. Schwartz
	Matthew J. Kennedy
	Jason Aoto
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Cover Letter

University of Colorado
Anschutz Medical Campus

Jason Aoto, Ph.D
Assistant Professor
University of Colorado, Denver
Department of Pharmacology

12800 E. 19th Ave, Room P18-6101
Aurora, CO 80045
Phone: (303) 724-9302
Email: Jason.aoto@ucdenver.edu

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The Editors
The Journal of Visualized Experiments

Dear Editors,

We are pleased to submit our manuscript entitled: **"Measuring Transsynaptic Interactions Through Protein Aggregation in a Heterologous Cell System"** for consideration for publication in The Journal of Visualized Experiments. Cell-to-cell communication often relies on the physical connection of adjoining cells generally mediated by surface proteins on each cell's membrane. Intercellular connections can lead to a multitude of biological responses ranging from synapse formation to cancer progression, as such *it is imperative to understand the protein-protein interactions responsible for modulating these cell interactions.* Many quantitative approaches exist to measure protein-protein interactions; however, these existing approaches do not typically test true *trans* interactions. In addition, the existing approaches can be costly, lengthy and require specialized training and equipment. Here we present a relatively rapid and inexpensive approach that measures cell-cell binding mediated exclusively by interactions in *trans* using a heterologous cell system. This method may be performed in a matter of minutes without the need for specialized equipment or training. Moreover, the efficiency of this HEK cell aggregation approach lends it to be used to rapidly screen the effects of a point mutation on one membrane protein on the *trans* binding ability to another protein.

To provide an example of the assay at work, we posed the question: is the interaction between two essential synaptic proteins affected by a point mutation? More specifically, is the *trans* interaction of Neurexin3α with LRRTM2 affected by the A687T point mutation identified in a patient diagnosed with intellectual disability and epilepsy?

We hope that you and the reviewers agree that this is a fundamentally important method. We would like to suggest the following scientists with expertise in synaptic cell-adhesion molecules: Garrett Anderson, David Martinelli and Bo Zhang as reviewers. Due to potential conflicts of interest, we respectfully ask that Jaewon Ko be excluded as a reviewer.

Sincerely,

Jason Aoto, Ph.D

TITLE:

Measuring Transcellular Interactions through Protein Aggregation in a Heterologous Cell System

AUTHORS AND AFFILIATIONS:

Susana Restrepo, Samantha L. Schwartz, Matthew J. Kennedy, Jason Aoto

Department of Pharmacology, University of Colorado Denver School of Medicine, Aurora, Colorado

CORRESPONDING AUTHOR:

Jason Aoto (Jason.aoto@cuanschutz.edu)

EMAIL ADDRESSES OF CO-AUTHORS:

Susana Restrepo (susana.restrepo@cuanschutz.edu)
Samantha L. Schwartz (samantha.schwartz@cuanschutz.edu)
Matthew J. Kennedy (matthew.kennedy@cuanschutz.edu)

KEYWORDS:

aggregation; synaptic ligands; HEK293T; trans protein interactions; Neurexin; LRRTM; Cell-adhesion

SUMMARY:

Here, we present an optimized protocol to rapidly and semiquantitatively measure ligand-receptor interactions in trans in a heterologous cell system using fluorescence microscopy.

ABSTRACT:

Protein interactions at cellular interfaces dictate a multitude of biological outcomes ranging from tissue development and cancer progression to synapse formation and maintenance. Many of these fundamental interactions occur in trans and are typically induced by heterophilic or homophilic interactions between cells expressing membrane anchored binding pairs. Elucidating how disease relevant mutations disrupt these fundamental protein interactions can provide insight into a myriad of cell biology fields. Many protein-protein interaction assays do not typically disambiguate between cis and trans interactions, which potentially leads to an overestimation of the extent of binding that is occurring in vivo and involve labor intensive purification of protein and/or specialized monitoring equipment. Here, we present an optimized simple protocol that allows for the observation and quantification of only trans interactions without the need for lengthy protein purifications or specialized equipment. The HEK cell aggregation assay involves the mixing of two independent populations of HEK cells, each expressing membrane-bound cognate ligands. After a short incubation period, samples are imaged and the resulting aggregates are quantified.

INTRODUCTION:

Synaptic interactions facilitated by synaptic adhesion molecules are foundational for the development, organization, specification, maintenance and function of synapses and the

generation of neural networks. The identification of these transsynaptic cell adhesion molecules is rapidly increasing; thus, it is fundamentally important to identify binding partners and understand how these new adhesion molecules interact with each other. Additionally, genome sequencing has identified mutations in many of these adhesion molecules that are commonly linked to a multitude of neurodevelopmental, neuropsychiatric, and addiction disorders[1]. Mutations in genes that code for synaptic cell-adhesion molecules may detrimentally alter trans interactions and may contribute to pathophysiological alterations in synapse formation and or maintenance.

Multiple assays exist to quantitatively assess protein-protein interactions such as isothermal calorimetry, circular dichroism, surface plasmon resonance[2] and although quantitative in nature, they have several limitations. First, they require recombinant protein, sometimes demanding lengthy and tedious purification steps. Second, they require sophisticated specialized equipment and technical expertise. Third, they can overestimate the extent of binding as they allow for both cis and trans interactions between proteins that are naturally tethered to a membrane in vivo. Here we propose a simple and relatively rapid assay that exclusively tests trans interactions.

To circumvent many of the complications associated with purified protein assays, we have optimized a cell-based protein interaction assay that recapitulates trans interactions in a reduced heterologous cell system. This assay has been previously used in various forms to study transcellular interactions. In this approach, candidate cell adhesion molecules are transfected into HEK293T cells. At physiological conditions, HEK293T cells do not exhibit self-aggregation, making them exemplary models for this assay. However, when individual populations of HEK cells expressing receptor and ligand are combined, the binding of the receptor and the ligand forces aggregation of HEK cells to occur. This aggregation is mediated exclusively by trans interactions and is usually observable in tens of minutes. No protein purification steps are required in this method, and the efficiency of the method relies on the paradigm that populations of HEK cells expressing cognate adhesion molecules are being combined and then imaged only tens of minutes later. Additionally, this method is relatively inexpensive, as neither antibodies nor costly equipment are required. The only equipment required for the acquisition of data is a standard fluorescent microscope. An additional advantage to this cell-based assay is the ability to quickly screen the effect of disease relevant point mutations on trans interactions. This can be performed by transfecting HEK cells with cDNAs of the mutant variants of the protein of interest.

In this protocol, we present an example in which we investigate whether a missense mutation in Neurexin3α (Neurexin3α^{A687T}), identified in a patient diagnosed with profound intellectual disability and epilepsy, alters interactions in trans with leucine-rich repeat transmembrane protein 2 (LRRTM2). Neurexin3α is a member of the evolutionarily conserved family of presynaptic cell-adhesion molecules and while recent work has identified multiple roles at the synapse[3,4,5,6,7], our synaptic understanding of this molecule and all members of the neurexin family remains incomplete. LRRTM2 is an excitatory postsynaptic cell adhesion protein that participates in synapse formation and maintenance[8,9,10]. Importantly, LRRTM2 exclusively interacts with neurexin isoforms that lack the splice site 4 alternative exon (SS4-) but not with neurexin isoforms containing the splice site 4 alternative exon (SS4+). The human missense

mutation (A687T) identified in Neurexin3α is located in an unstudied extracellular region that is evolutionarily conserved and is conserved between all alpha neurexins[7]. As the interaction between these two molecules has been established[8,9,11], we posed the question: is the binding capability of Neurexin3α SS4- to LRRTM2 altered by an A687T point mutation? This assay revealed that the A687T point mutation unexpectedly enhanced the aggregation of Neurexin3α to LRRTM2 suggesting that the extracellular region in which the point mutation is located, plays a role in mediating transsynaptic interactions.

PROTOCOL:

1. Cell culture and transfection

1.1. Make HEK cell media with DMEM, 1x (Dulbecco's Modification of Eagle's Medium) supplemented with 4.5 g/L glucose, L-glutamine & sodium pyruvate and 10% FBS. Sterile filter.

1.2. Predetermine suitable ligands and receptors for aggregation assay.

NOTE: Neurexin3α SS4+/- and one of its known ligands, LRRTM2, were used in this study. Ligands and receptors of interest were expressed from cDNAs in pcDNA3.1. A Gibson assembly was used to insert Neurexin3α into pcDNA3.1[12].

Neurexin3α F/R: TTTAAACTTAAGCTTGGTACCGAGCTCGGATCCGCCACCATGAGCTTTACCCTCCACTC/GAGCGGCCGC CACTGTGCTGGATATCTGCAGAATTCTTACACATAATACTCCTTGTCCTT.

1.3. Prepare HEK293T cells.

1.3.1. Grow HEK293T cells to confluency in one T-75 flask.

1.3.2. Once confluent, use 2 mL of trypsin and place in 37 °C incubator for 2 min. Add 6 mL of HEK media to the flask to resuspend cells and transfer all 8 mL to a 15 mL conical tube.

1.3.3. Pellet at 500 x *g* for 5 min and resuspend in HEK cell media for a total of 8 mL.

1.3.4. Count cells and add 735,000 cells into each well of a 6-well plate. Adjust final volume to 2 mL for each well using HEK cell media.

1.3.5. Place in 37 °C incubator and allow cells to grow overnight or until they reach 50-60% confluency.

1.4. Transfect HEK293T cells using the calcium phosphate method[13].

1.4.1. Transfect well-1 with 3 µg of the protein of interest and co-transfect with 1 µg of fluorescent protein (3 µg of pCDNA3.1-Neurexin3α^{WT} SS4- and 1 µg of mCherry).

1.4.2. Transfect well-2 as in step 1.4.1. but with the mutated protein of interest (pCDNA3.1-Neurexin3α^{A687T} SS4-).

1.4.3. Transfect well-3 with 3 µg of the ligand of interest and co-transfect with 1 µg of another fluorescent protein (3 µg of pCDNA3.1 LRRTM2 and 1 µg of GFP).

1.4.4. Transfect well-4 and well-5 to serve as negative controls: well-4 with 1 µg of GFP and well-5 with 1 µg of mCherry.

1.4.5. Prepare another plate (as in step 1.4.1-1.4.4) if requiring additional conditions or controls (Neurexin3$\alpha^{WT/A687T}$ SS4+).

NOTE: Transfection efficiency is analyzed 24 h after transfection under an epifluorescence microscope and quantified as the number of cells expressing the fluorescent protein they were transfected with. A more streamlined approach would include the transfection of HEK cells with a bicistronic vector coding for a fluorescent protein and the ligand of interest and is highly recommended above co-transfection. In the case of this study, alpha Neurexins are ~4.3 kb and low fluorescence intensity was observed using a bicistronic system necessitating co-transfection.

1.5. 48 hours after transfection, harvest cells for aggregation.

1.5.1. Wash each well twice with PBS.

1.5.2. Add 1 mL of 10 mM EDTA in PBS into each well to gently dissociate cell-to-cell interactions and incubate plate at 37 °C for 5 min.

NOTE: Trypsin is not recommended for step 1.5.2 due to potential proteolytic cleavage of adhesion molecules in study. Additionally, after EDTA addition the protocol may not be stopped until completion as cells will now be exposed to ambient conditions.

1.5.3. Gently tap plate to detach the cells, and harvest each well into separate 15 mL conical tubes.

1.5.4. Centrifuge conical tubes at 500 x *g* and room temperature for 5 min.

1.6. While cells are pelleting, prepare 6 incubation tubes by labeling the top of each microcentrifuge tube with each condition.

NOTE: Each permutation of GFP and mCherry conditions should be used to encompass all experimental conditions and proper controls. For example: 1. GFP/mCherry, 2. mCherry/LRRTM2-GFP 3. GFP/Neurexin3α^{WT} SS4-—mCherry, 4. GFP/Neurexin3α^{A687T} SS4- –mCherry, 5. Neurexin3α^{WT} SS4-—mCherry/LRRTM2—GFP, 6. Neurexin3α^{A687T} SS4- –mCherry/LRRTM2—GFP. Make additional tubes to accommodate further conditions and

controls.

1.7. Remove the supernatant and resuspend cells in 500 µL of HEK media with 10 mM $CaCl_2$ and 10 mM $MgCl_2$ warmed to 37 °C.

NOTE: The addition of $CaCl_2$ and $MgCl_2$ allows adhesion molecules to reestablish binding and is only required if the transcellular interaction partners in question require divalent cations for adhesion.

1.8. Count the cells in each 15 mL conical tube using a hemocytometer and aliquot 200,000 cells of each condition into appropriate tube from step 1.6.1 for a 1:1 mix in a total volume of 500 µL.

NOTE: It should only take 5 min per condition to count and aliquot amounts.

1.9. Incubate tubes at room temperature in a slow tube rotator.

2. Image acquisition

2.1. Optimize microscope acquisition parameters for specific samples. In this example, images were taken on a wide-field microscope. Use a 5x air objective (NA: 0.15; WD: 20000 µm) to get a large enough field for analysis.

2.2. Assess baseline aggregation immediately after mixing the two conditions of HEK cells in step 1.8. These are now the 'time zero' images.

2.2.1. Pipette 40 µL of each sample mixture onto a charged microscope slide and image under fluorescence in both the 488 and 561 channels.

2.2.2. Acquire three different fields of view at one focus plane per sample drop.

2.3. Acquire final images at 60 min as the 'time 60' image.

2.3.1. To obtain the 'time 60' image of the mixture after a 60 min incubation, take another 40 µL sample of each condition from rotating tubes and pipette each sample onto a charged slide. Image as in step 2.2.2.

NOTE: Cell aggregation should be checked every 15 min until saturation occurs. Timing of aggregation will depend on the proteins being tested.

3. ImageJ/Fiji Analysis

3.1. To quantify the extent of aggregation using Fiji/ImageJ, save analysis files.

3.1.1. Save the provided Supplemental coding files into the imageJ macros folder on the

computer.

3.1.2. Install the aggregation macro provided (Plugins, Macros, Install, and select the "AggregationAssay.txt" file).

3.2. Determine thresholds.

3.2.1. Load a 'time zero' .tif file into imageJ and split the channels (**Image | Color | Split Channels**).

NOTE: The 'time zero' image is used to determine the thresholding and smallest puncta size for the whole experiment.

3.2.2. Mask each channel (**Plugins | Macros | AggregationAssay_MakeMask**). **Make Mask From Image** window will appear. Check boxes next to **Determine Threshold for Image** and **Determine Cluster Params from Histogram** and click **OK**.

3.2.3. Determine the threshold of the image using the slide bar, record the number to the right of the slide bar and click **OK**.

3.2.4. A **Histogram of Cluster Size** will appear. Select a cluster size from the histogram that suits the experiment, type this number in the **Min Cluster Size:** box, and click **OK**. Clusters below this size will not be analyzed.

3.3. Run the analysis.

3.3.1. Open the 'time 60' image of condition 1 in imageJ and split the channels as in step 3.2.1.

3.3.2. Mask each channel (Plugins, Macros, AggregationAssay_MakeMask). Use the same threshold and size determined in step 3.2.3 and step 3.2.4. Unselect the boxes next to **Determine Threshold for Image** and **Determine Cluster Params from Histogram** and manually type the size and thresholds into the appropriate fields then click **OK**.

3.3.3. Calculate the aggregation index (**Plugins | Macros | AggregationAssay_CalculateOverlap**). Select the masked channels to be compared and directory into which the resulting files will save.

3.3.4. Repeat steps 3.3.1–3.3.3 for every 'time 60' image in every condition.

NOTE: The aggregation index is defined as the total overlap area divided by the sum of the two channel areas minus the overlap area multiplied by 100 (Aggregation index = overlap area/[area of channel 1 + area of channel 2 – overlap area] x 100). This normalization is an 'OR' operation between the two masked channels representing the total pixels in either mask.

REPRESENTATIVE RESULTS:

The A687T mutation increases Neurexin3a SS4- binding to LRRTM2[7]

To investigate how intercellular interactions of two known synaptic proteins are affected by the introduction of a point mutation found in a patient with intellectual disability and epilepsy, we used the above HEK cell aggregation assay (**Figure 1**). Cells were transfected according to section 1 and prepared for imaging according to sections 1 and 2 of the protocol. Cells were imaged at baseline where no aggregation was observed as expected (not shown). Images acquired at 60 minutes were analyzed as in section 3 of the protocol. To minimize selection bias, conditions were randomized to blind the experimenter. For similar reasons the whole field of view of every image was selected as an ROI.

Conditions in which cells were not expressing any synaptic ligands (GFP/mCherry) showed minimal aggregation after a 60-minute incubation (**Figure 2**). Equally, conditions in which only one of the two populations of cells were expressing synaptic ligands (mCherry/LRRTM2-GFP or GFP/Neurexin3$\alpha^{WT/A687T}$ SS4- —mCherry) exhibited minimal aggregation after a 60 min incubation (**Figure 2**). As expected, conditions that contained two populations of cells with incompatible adhesions molecules (Neurexin3$\alpha^{WT/A687T}$ SS4+—mCherry/LRRTM2-GFP) exhibited no aggregation at 60 minutes (**Figure 2B**). These conditions served as critical negative controls as LRRTM2 is known to bind exclusively to SS4 lacking isoforms (SS4-) of Neurexins and highlights the specificity of this aggregation assay.

Conditions with compatible adhesion molecules[8,9,10] (Neurexin3a^{WT} SS4- —mCherry/LRRTM2-GFP) exhibited significant aggregation after a 60 min incubation (**Figure 2C**). Aggregation can be visualized as yellow and is present in the overlap between cells (**Figure 1** and **Figure 2**). Surprisingly, the condition where Neurexin3α A687T SS4- was co-incubated with LRRTM2 (Neurexin3α^{A687T} SS4- —mCherry/LRRTM2-GFP) exhibited significantly more aggregation as compared to its wildtype counterpart (Neurexin3α^{WT} SS4- —mCherry/LRRTM2-GFP; positive control). This suggests that the A687T point mutation in Neurexin3α enhances the binding capabilities of Neurexin3α SS4- to LRRTM2.

FIGURE AND TABLE LEGENDS:

Figure 1. Workflow of aggregation assay. (**A**) HEK293T cells are transfected using a protein-1/mCherry, or a protein-2/GFP. (**B**) Expression of Neurexin3α takes 48 h. (**C**) Cells are harvested using 10 mM EDTA and then pelleted (**D**). (**E**) Cells are mixed in a 1:1 ratio and resuspended in 500 μL of HEK media containing 10 mM $CaCl_2$ and $MgCl_2$. (**F**) Cells are incubated at room temperature until aggregation occurs and a final image is acquired at 'time 60'. Aggregation is visualized as the number of yellow puncta visible in the field of view. No aggregation is observed when cells are not expressing the correct receptor ligand pairs and are thus seen as individual red or green puncta.

Figure 2. Neurexin3α^{A687T} SS4- has enhanced aggregation with excitatory ligand LRRTM2 compared to Neurexin3α^{WT} SS4-. (**A**) Representative images of negative controls after a 60 min incubation of mCherry with GFP, mCherry with LRRTM2, GFP with Neurexin3α^{WT} SS4+/-, and GFP with Neurexin3α^{A687T} SS4+/-. Aggregation was observed after 60 minutes in both LRRTM2 with

Neurexin3α^{WT} SS4-, and LRRTM2 with Neurexin3α^{A687T} SS4-. (**B**) Quantification of the aggregation index in all SS4+ conditions after 60 minutes. Aggregation index = overlap area/(area of channel 1 + area of channel 2 – overlap area) x 100. (**C**) Same as (**B**) but SS4-. Data shown represent the mean ± SEM of the number of experiments (SEM: GFP/mCherry ± 0.0245, mCherry/LRRTM2 ± 0.02465, GFP/Neurexin3α^{WT} SS4- ± 0.02453, GFP/Neurexin3α^{A687T} SS4- ± 0.0109, Neurexin3α^{WT} SS4-/LRRTM2 ± 0.0538, Neurexin3α^{A687T} SS4-/LRRTM2 ± 0.0174; p= 0.0136). Numbers presented in bars represent the number of independent experiments carried out. Dotted lines represent baseline or average minimal aggregation of control conditions. Statistical significance was determined by one-way ANOVA, multiple comparisons; *p_0.05; ****p_0.0001.This figure has been modified from Restrepo et al.[7].

DISCUSSION:

Dissecting the protein-protein interactions that occur in trans during cell adhesion can lead to a better understanding of the molecular mechanisms underlying basic cellular processes including the formation, function and maintenance of synapses during maturation and remodeling. The implications of cell-to-cell interactions expand beyond neurobiology and have broader roles in signal transduction, cell migration and tissue development[14]. Aberrations in cell adhesion can disrupt cellular processes imperative for proper cell function and can underly a variety of etiologies such as cancer, arthritis, addiction, autism and schizophrenia[1,15,16]. Here, we provide an optimized detailed protocol involving HEK cell aggregation that allows for testing cell-adhesion interactions in trans.

With this HEK cell aggregation protocol, we can dissect differences in aggregation to approximate binding capacity between a presynaptic protein and its postsynaptic ligand. As such, we can ask questions such as: is the interaction between two essential synaptic proteins affected by a point mutation? More specifically, is the trans interaction of Neurexin3α with LRRTM2 affected by A687T? The A687T missense mutation studied here is located in a previously unstudied linker region of the extracellular domain of Neurexin3α[7]. The results illustrate that the A687T mutation enhances the aggregation of cells containing Neurexin3α^{A687T} to cells containing LRRTM2 (**Figure 2C**). This finding is significant because it was previously shown that LRRTMs only bind to Neurexins via a Neurexin domain called LNS6[17], and further suggests that sequences upstream of LNS6 can exert an independent effect on the trans interactions between Neurexin3α SS4- and LRRTM2[7].

The results in **Figure 2** exhibit the specificity of this assay as all negative controls (GFP/mCherry; Neurexin3$\alpha^{WT/A687T}$ SS4- —mCherry/GFP or LRRTM-GFP/mCherry) had no observable aggregation after 60 minutes. A critical control used in this study, Neurexin3$\alpha^{WT/A687T}$ SS4+ —mCherry/LRRTM-GFP, also exhibited no aggregation after 60 min because LRRTM2 is known to bind exclusively to SS4 lacking (SS4-) isoforms of Neurexin. This control demonstrates that simple overexpression of membrane-bound molecules is insufficient to force aggregation in this system, which further illustrates the specificity of this assay.

The cell adhesion assay described here has been widely used to test the interactions of transsynaptic cell-adhesion molecules[8,18,19]; however, it may be used to test adhesive cell-to-cell interactions of membrane tethered proteins. This protocol, which has evolved over time, was optimized from the original published protocol and subsequent variations of the protocol by changing three experimental parameters. One, the incubation temperature for the cells was changed. The original protocol calls for the incubation of cells in step 1.9 at 4 °C. This low temperature can act as an environmental stressor and can decrease cell viability leading to cell death and lysis. During cell lysis, the cells secrete genomic DNA and cell debris into media that causes cells to clump in solution leading to false positives during imaging. Two, the number of cells per condition were increased to 200,000 per population and the objective size was changed to a 5x; this allows the researcher to image a greater number of interactions per field of view in order to increase statistical power within each n. Three, the aggregation index was measured differently. Previous aggregation indices were limited by the selection of cluster size by the experimenter leading to the exclusion of "subthreshold" clusters. By contrast thresholds are now set for individual cell size at 'time zero', and the aggregation is now calculated as the overlapping area divided by the sum of the two channel areas minus the overlap area multiplied by 100 at 'time 60' which allows for the inclusion of smaller positive clusters making the assay more sensitive to other protein-ligand pairs (**Figure 2B,C**).

Troubleshooting and optimization may be needed depending on the interacting proteins tested. Although the incubation period until final image acquisition will differ depending on the tested proteins, it is critical that no aggregation is observed at 'time zero'. If aggregation is seen at time zero, it may imply that the cells were not healthy to begin with. This may be due to several factors including sudden exposure to temperatures below 37 °C or slow experimental procedure. Importantly, the resuspension media for step 1.7 should be at 37 °C, which will allow the cells to gradually reach room temperature without a sudden harsh drop in temperature. One way to have optimal cell health throughout the experiment is to ensure that steps 1.7 through 1.9 are completed within a 25 min timeframe with no breaks in between. It is recommended that the microscope is setup and ready to use before cell harvest cells in step 1.5; this ensures that a true 'time zero' image can be taken immediately in step 2.2.

If aggregation is not observed after 60 minutes of incubation, several factors may be contributing to this lack of adhesion. First, the proteins in question may not be binding partners or may engage in low affinity interactions not detectable by this assay. In this case, a more sensitive assay may be required. Currently the range of binding affinities this assay is sensitive to is unknown. Second, the protein(s) of interest may not localize to the membrane when expressed alone in HEK cells. In this case, confirm that the protein is membrane localized by using biochemical techniques (surface biotinylation) or directly tag the protein of interest with a fluorescent tag and image HEK293T cells at 40x or higher magnification to assess surface expression. However, after membrane localization is confirmed, we recommend using untagged variants for this HEK cell aggregation assay in order to more accurately assess the binding capacity of the native protein. Third, in this protocol we allowed Neurexin3α to express for 48 h; however, protein expression time may vary depending on the proteins tested. Fourth, it is critical to supplement the media in step 1.7 with $MgCl_2$ and $CaCl_2$ if the proteins in question are dependent on divalent cations as is

the case with the example of Neurexins and LRRTM2[11]. If it is unknown whether the interaction partners require divalent cations for adhesion, add EDTA into one condition. The EDTA should chelate remining cations naturally present in DMEM ensuring a calcium and magnesium free solution. If adhesion is observed after EDTA addition, the proteins in question do not require divalent cations for adhesion.

Many methods exist to test protein interactions; however, most are not specific for testing only trans interactions that occur from cell to cell and require tedious protein purification steps. Although the HEK cell aggregation assay is not a direct measure of affinity and should not be used to replace a more quantitative approach to recover dissociation constants, to the best of our knowledge, this assay represents an approach to test true trans interactions in a semi-quantitative and relatively efficient manner. Moreover, due to the relative ease of this assay, the HEK cell aggregation assay can be used in conjunction with these more quantitative approaches to obtain a broader and more complete characterization of the interactions taking place.

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DISCLOSURES:

The authors have nothing to disclose.

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15. Burdick, M.M., McCarty, O.J.T., Jadhav, S., Konstantopoulos, K. Cell-cell interactions in inflammation and cancer metastasis. *IEEE Engineering in Medicine and Biology Magazine*. **20** (3), 86–91 (2001).
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19. Boucard, A.A., Ko, J., Südhof, T.C. High Affinity Neurexin Binding to Cell Adhesion G-protein-coupled Receptor CIRL1/Latrophilin-1 Produces an Intercellular Adhesion Complex. *Journal of Biological Chemistry*. **287** (12), 9399–9413 (2012).

Figure 1

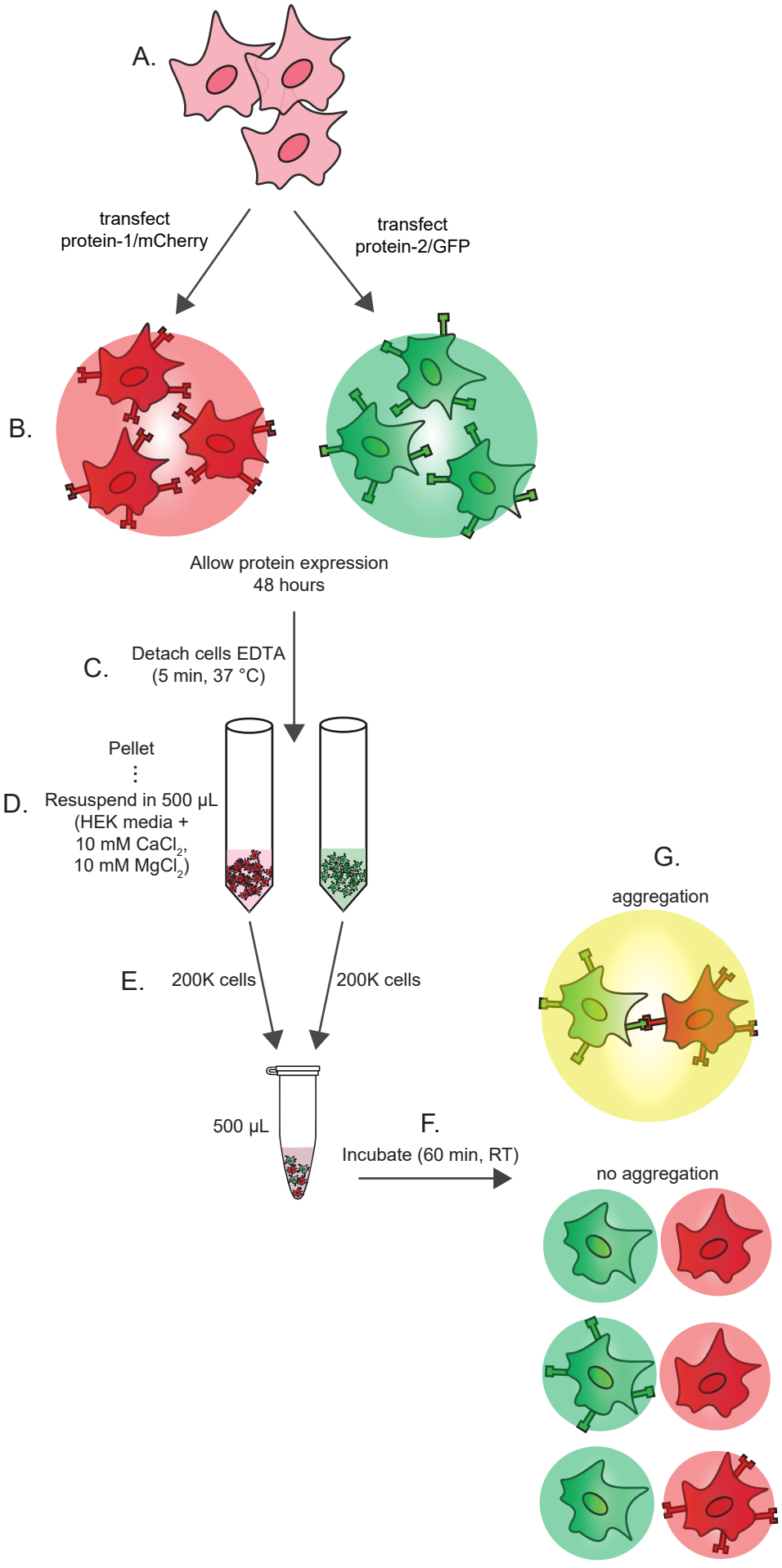
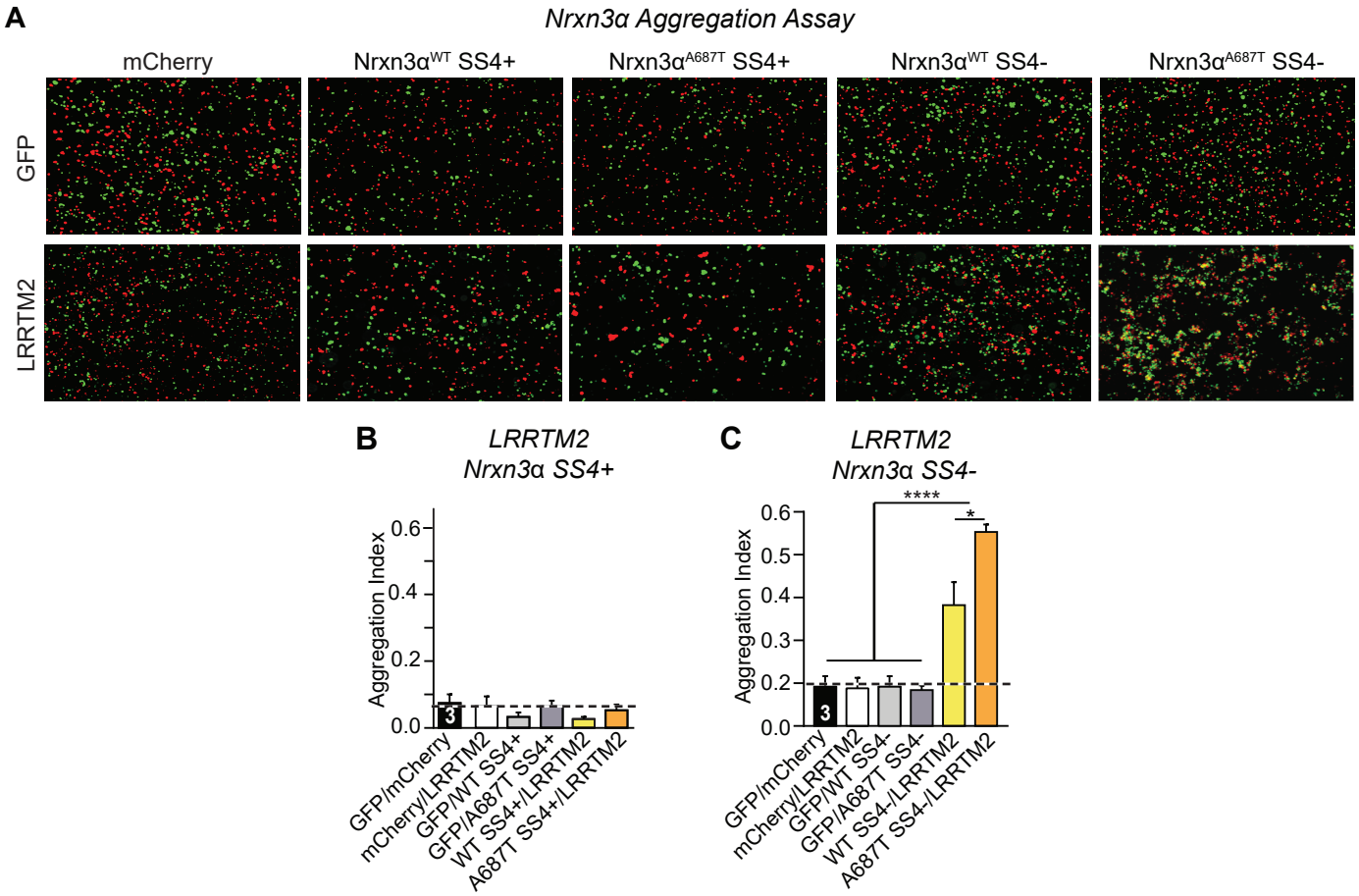


Figure 2

Click here to access/download;Figure;Figure_2.ai



Name of Material/Equipment	Company	Catalog Number	Comments/Description
1.5 mL disposable microtubes with snap caps	VWR	89000-028	Incubation of mixed population of HEK cells
1000 mL Rapid—Flow Filter Unit, 0.2 um aPES membrane	Thermo Fisher	567-0020	Sterilization of HEK media
15 mL SpectraTube centrifuge tubes	Ward's Science	470224-998	Harvesting HEK cells
6 well sterile tissue culture plates	VWR	100062-892	culturing HEK cells
Calcium Chloride	Sigma	223506-500G	Calcium phosphate transfection, HEK cell resuspension
Centrifuge- Sorvall Legend RT	Kendro Laboratory Products	75004377	Harvesting HEK cells
CO2 cell incubator	Thermo Scientific	HERACELL 150i	Incubation of HEK cells
DMEM, 1x (Dulbecco's Modification of Eagle's Medium) with 4.5 g/L glucose, L-glutamine & sodium pyruvate	Corning	10-013-CV	HEK cell maintenance
Dulbecco's Phosphate Buffered Saline PBS (1X)	Gibco	14190-144	Passaging/harvesting HEK cell
Ethylenediaminetetraacetic acid	Sigma	ED-500G	Harvesting HEK cells
Falcon Vented culture flasks, 75cm^2 growth area	Corning	9381M26	Culturing HEK cells
Fetal Bovine Serum	Sigma	17L184	HEK cell maintenance
HEK293T cells	ATCC		Model system
ImageJ	NIH	V: 2.0.0-rc-69/1.52p	Image analysis
Magnesium Chloride hexahydrate	Sigma	M9272-500G	HEK cell resuspension
Sodium phosphate dibasic anhydrous	Fisher BioReagents	BP332-500	Calcium phosphate transfection
Trypsin 0.25% (1X) Solution	GE Healthcare Life Sciences	SH30042.01	Passaging HEK cells

Tube rotator			Incubation of mixed population of HEK cells
UltraClear Microscope slides. White Frosted, Positive Charged	Denville Scientific Inc.	M1021	Image acquisition
Wide-field microscope	Zeiss	Axio Vert 200M	Image acquisition

Authors' Response to the Reviewers' Comments for Restrepo et al., "**Measuring Transcellular Interactions Through Protein Aggregation in a Heterologous Cell System**", and Changes Instituted in the Revised Manuscript

We would like to thank the editor and reviewers for their time reviewing this manuscript and for their valuable and helpful comments. For the revision we have added the reviewers' suggestions and feel we have addressed all concerns. We hope that with the additions and the changes made in the revised manuscript as described in detail below, the reviewers will find the paper acceptable for publication, and would like to thank the reviewers and editors for their consideration.

Editor:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We would like to thank the editor for their time reviewing this manuscript. We have gone over the manuscript again and corrected several mistakes (see tracked changes on manuscript).

2. Please provide at least 6 key words or phrases.
We have now provided 7 key words and phrases (aggregation; synaptic ligands; HEK293T; trans protein interactions; Neurexin; LRRTM; Cell-adhesion).

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: UltraClear
We have removed 'UltraClear', 'Zeiss Axio Vert' and 'Invitrogen' from the main text.

Protocol:
1. For each protocol step/substep, 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. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.
We have added a few key phrases that will further instruct the reader how to perform certain steps (see tracked changes on manuscript). We believe the rest reflects the adequate amount of 'how' information for the common scientific reader to carry out the described experiment.

Specific Protocol steps:
1. 1.3.2: Should there be a pelleting step before this one?
We have added this step as part of 1.3.2 and added further detail that connects to 1.3.1 to 1.3.2.: "….Once confluent, use 2 ml of Trypsin and place in 37 °C incubator for 2 minutes. Add 6 ml of HEK media to the flask to resuspend cells and transfer all 8 ml to a 15 ml conical tube…. 1.3.2. Pellet at 500 x g for 5 minutes and resuspend in HEK cell media for a total of 8 ml."

Figures:
1. Please cite Figure 1 before Figure 2.
We have now cited Figure 1 before Figure 2: "To investigate how intercellular interactions of two known synaptic proteins are affected by the introduction of a point mutation found in a patient with intellectual disability and epilepsy, we used the above HEK cell aggregation assay (Figure 1)."

2. Figure 2A: Are the GFP and LRRTM2 labels switched? Also, a GFP with LRRTM2 image does not appear to be here.
We thank the editor for catching this egregious mistake on our part. The GFP and LRRTM2 labels *were* switched and have now been rectified. The "GFP with LRRTM2" was a typo on the original submission. There should not be a GFP with LRRTM2 condition as GFP was co-transfected with LRRTM2. The figure legend for Figure 2 now reflects these changes.

3. Figure 2B,C: What do the dotted lines and asterisks signify? Why is there a '3' only in the GFP/mCherry bars? Lastly, there are two 'SS4+' labels in C; it appears those should be 'SS4-'.
We apologize for the oversight that led to the exclusion of this important information in the original text. The dotted lines on each bar graph represent baseline or average minimal aggregation of control conditions and the '3' on the first bar of each graph is meant to indicates that for every condition across the bar graph three independent experiments were carried out. We found that adding this dotted line to represent minimal aggregation aids in visual comprehension because these assays often have many bars associated with the various controls. These clarifications are now present in the Figure 2 legend. The 'SS4+' label in C was incorrect and has now been amended in the figure.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.
We believe the table of materials reflects all the required materials for this study.

Reviewer #1:
Manuscript Summary:
In this manuscript by Restrepo et al., the authors report an optimized protocol about measuring transsynaptic interactions through protein aggregation assay in a heterologous cell system. This method paper is based on a very thoroughly-conducted and interesting paper published in Journal of Neuroscience from Dr. Aoto's lab (Restrepo et al., 2019, J Neurosci). This method paper is well-written with a balanced introduction and discussion and should be accepted in the current form.
We thank the reviewer for all the positive comments.

Minor Concerns: Minor spell error in line 103: "1.1.2" should be "1.1.1".
We have corrected this mistake.

Reviewer #2:
Manuscript Summary:
In this manuscript, the authors describe an optimized version of a previously described method for trans-directed protein interactions in a heterologous cell system that was used in different variations from the original published protocol, before. Three major parameters were changed: the incubation temperature (from 4°C to room temperature), number of cells used for aggregation was increased by a factor of five, and the definition of background aggregation.
We thank the reviewer for all the constructive feedback on this manuscript.

Major Concerns:
The manuscript describes a previously unpublished unexpected finding for a mutation in Neurexin 3alpha. While the authors present this correctly, I am concerned that this paper will be cited later with regard to this unexpected finding (requiring more validating data) and not as a methodological article explaining the protocol in detail. I therefore recommend to remove this finding from the manuscript.
We thank the reviewer for their concern. The finding presented in this manuscript was previously published by Restrepo *et al.*, "Modeling a Neurexin-3α Human Mutation in Mouse Neurons Identifies a Novel Role in the Regulation of Transsynaptic Signaling and Neurotransmitter Release at Excitatory Synapses," *The Journal of Neuroscience,* 2019. This paper is cited throughout the manuscript as citation #7 including in the introduction, Figure 2 legend, and the discussion section; however, we agree that the citation was missed in some sentences related to the findings. We have thus added the citations to these sentences as well:

- **A. "This finding is significant because it was previously shown that LRRTMs only bind to Neurexins via a Neurexin domain called LNS6[1], and further suggests that sequences upstream of LNS6 can exert an independent effect on the *trans* interactions between Neurexin3α SS4- and LRRTM2[2]."**
- **B. "REPRESENTATIVE RESULTS: The A687T mutation increases Neurexin3a SS4- binding to LRRTM2[7]"**

Minor Concerns:
Title: "transsynaptic" should be changed to "transcellular" as the assay does not reflect a synaptic situation.

We wholeheartedly agree with the reviewer's comment and have changed the title to "Measuring Transcellular Interactions Through Protein Aggregation in a Heterologous Cell System".

Introduction (line 55 to 61): First, Two, Three should be changed to First, Second, Third.
We thank the reviewer for their great suggestion, and we have now changed "First, Two Three" to "First, Second, Third".

Introduction: The authors should indicate in the introduction that the assay has been used in different variations before.
We agree with the reviewer's comment and have now incorporated the following into the introduction: "This assay has been previously used in various forms to study transcellular interactions.".

Protocol:
Step 1.3.3: give the cell number
We thank the reviewer for their comment and agree that it is best practice to state a cell number rather than a volume. We have now added the cell number to this step.

Step 1.4.3: co-transfection with individual plasmids is not state of the art. Instead of this, bicistronic vectors or fusionproteins with 2a self cleaving peptide sites should be recommended.
We agree and thank the reviewer for their point about using a better approach for co-expression. We have added the following to the text: "A more streamline approach would include the transfection of HEK cells with a bicistronic vector coding for a fluorescent protein and the ligand of interest and is highly recommended above co-transfection. In the case of this study, alpha Neurexins are ~4.3 kb and low fluorescence intensity was observed using a bicistronic system necessitating co-transfection."

Step 1.5.2: Background to addition of EDTA and later on addition of Ca and Mg should be explained
We thank the reviewer for bringing attention to this point, and we agree that this information is necessary especially in a methods paper. We have thus added the following to the text:

A. **"Add 1 ml of 10 mM EDTA in PBS into each well to gently dissociate cell-to-cell interactions and incubate plate at 37 °C for 5 minutes.**
   NOTE: Trypsin is not recommended for step 1.5.2 due to potential proteolytic cleavage of adhesion molecules in study…."

B. **"Vacuum out supernatant and resuspend cells in 500 ul of HEK media with 10 mM $CaCl_2$ and10 mM $MgCl_2$ warmed to 37 °C.**
   NOTE: The addition of $CaCl_2$ and $MgCl_2$ allows adhesion molecules to reestablish binding and is only required if the transcellular interaction partners in question require divalent cations for adhesion."

Discussion
The authors should state on the addition of Ca and Mg in step 1.7. Is it recommended for trans cellular interactions that are independent of Ca and or Mg?
We thank the reviewer for their helpful comment, and we agree that it is necessary to clarify this point. We have added the following to the discussion text: "Fourth, it is critical to supplement the media in step 1.7 with $MgCl_2$ and $CaCl_2$ if the proteins in question are dependent on divalent cations as is the case with the example of Neurexins and LRRTM2[3]. If it is unknown whether the interaction partners require divalent cations for adhesion, add EDTA into one condition. The EDTA should chelate remining cations naturally present in DMEM ensuring a calcium and magnesium free solution. If adhesion is observed after EDTA addition, the proteins in question do not require divalent cations for adhesion."

Figure 1
The figure should represent more critical steps of the workflow, such as EDTA treatment, Ca/Mg incubation, cell number/volume, time of incubation.
We agree with the reviewer that the figure should in more detail describe critical steps of the protocol. We have now expanded the figure (see right) and respective figure legend to include this detail:
"Figure 1. Workflow of aggregation assay. (A.) HEK293T cells are transfected using a protein-1/mCherry, or a protein-2/GFP. (B.) Expression of Neurexin3a takes 48 hours. (C.) Cells are harvested using 10 mM EDTA and then pelleted (D.). (E.) Cells are mixed in a 1:1 ratio and resuspended in 500 ul of HEK media containing 10mM $CaCl_2$ and $MgCl_2$. (F.) Cells are incubated at room temperature until aggregation occurs and a final image is acquired at 'time 60'. Aggregation is visualized as the number of yellow puncta visible in the field of view. No aggregation is observed when cells are not expressing the correct receptor ligand pairs and are thus seen as individual red or green puncta."

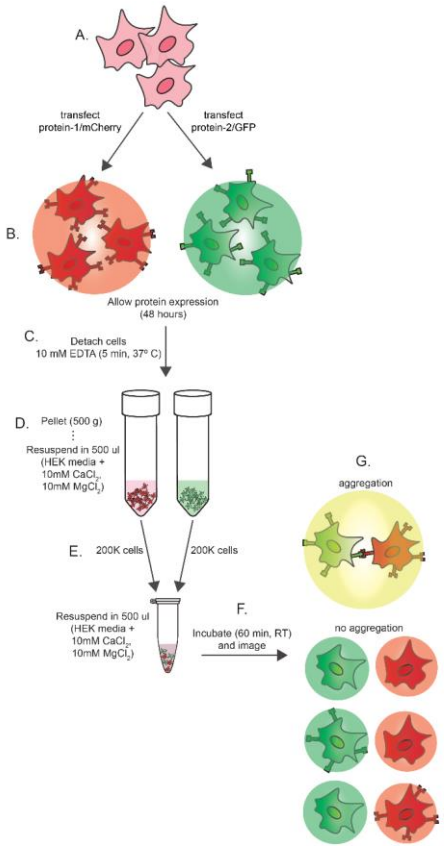


Figure 2
- The aggregation index should be explained in more detail.
We have added a definition to aggregation index in step 3.3, to the Figure 2 legend and in the discussion.

- Labeling in Fig 1c is wrong. It should be SS4-
We thank the reviewer for catching this glaring mistake and we have now corrected it to "SS4-".

Reviewer #3:
Manuscript Summary:
Restrepo et al. present a simple assay to measure trans interactions between membrane proteins. The protocol is well explained and easy to use, and will be of interest to many biologists working on cell-cell interactions. I have only the following minor points:
We thank the reviewer for their time reviewing this manuscript and appreciate the positive and beneficial comments.

Major Concerns:
None

Minor Concerns:
P6 image acquisition: it was not entirely clear to me whether at 60 minutes, a new 40ul of sample mixture is spotted on the microscope slide, or whether the same 40 ul drop from time zero is imaged again (I suspect it is a new one taken from the rotating tube). Please clarify.
We thank the reviewer for bringing to light this important clarification that needed to be made. We have now stated: "To obtain the 'time 60' image of the mixture after a 60 minute incubation take another 40 ul sample of each condition from rotating tubes and pipette each sample onto a charged slide. Image as in 2.2.2."

P9 line 324: 'may be used to test any cell-to-cell interactions that involve membrane-tethered proteins'- the assay to me

seems mainly to apply to those trans interactions that are adhesive; not all interactions that occur in trans may have this effect. Please discuss.

The reviewer's comment raises two interesting points regarding adhesive and non-adhesive transmembrane proteins that we would like to briefly discuss here:

1. The line separating adhesion molecules from "non-adhesion" transmembrane molecules is becoming blurred and functional classification seems to be dependent on the system studied. For example, Notch-delta interactions are commonly classified as signal transducing interactions and are thus frequently studied in that context. However, there is evidence that Notch-delta can function as adhesion molecules[4,5]. Additionally, the Toll receptor, largely considered to be a signal-transducing, non-adhesive transmembrane protein, functions in drosophila as an adhesive molecule[6]. Importantly, these two non-adhesive transmembrane proteins have been shown to induce aggregation in similar, but unoptimized, heterologous cell aggregation assays, which suggests that trans-interactions, independent of whether the molecules are adhesive or not, can be monitored using this assay[7,6].

2. Additionally, classic adhesion molecules such as Neurexins that adhere to their ligands are highly dynamic in nature[8]. Yet, despite the high dynamicity of Neurexin interaction, we are still able to capture the essence of the interaction with this assay. Previous studies using various forms of this assay have shown all-or-nothing interactions[9]. Here we show the sensitivity of the assay by showing how single point mutation modulates the interaction between ligand-protein pairs and even enhances it. Other transmembrane proteins known to initiate intracellular signaling cascades still require interactions at the cell surface however transient in nature[10].

To clarify the statement in question, we have amended the manuscript to: "it may be used to test adhesive cell-to-cell interactions of membrane tethered proteins"

P10 line 359: the interaction of neurexin-1a with LRRTM2 using purified protein is mentioned to be around 20 nM. If I interpret the text correctly, the authors seem to suggest that this is in the lower range of affinities and may therefore require a more sensitive assay than the one presented here. Is the affinity of neurexin-1a for LRRTM2 lower than the affinity of neurexin-3a for LRRTM2? Affinity measurements obtained using purified proteins may differ from those obtained on the cell surface. In addition, 20 nM is still a relatively high affinity for cell surface interactions; many surface interactions are in the 100s of nM to uM range. What is the range of binding affinities for which the assay described here can be used? Please clarify this point.

We thank the reviewer for their valuable comment and upon reflection realized that the wording of this paragraph was confusing to begin with. The original arrangement of sentences seemed to suggest that that the assay would not be sensitive to 20 nM affinities and below. However, the 20 nM affinity was meant as an example for the reader to have a frame of reference to the binding affinity of the LRRTM2 being tested to Neurexin1, a protein with high sequence identity to Neurexin3. Since we agree with the reviewer's earnest comment that "Affinity measurements obtained using purified proteins may differ from those obtained on the cell surface" we have removed that sentence from the text and apologize for the confusion. To further make the text more coherent, we have now moved the sentence "In this case, a more sensitive assay may be required" to logically follow the sentence "First, the proteins in question may not be binding partners or may engage in low affinity interactions not detectable by this assay." Additionally, we have added "Currently the range of binding affinities this assay is sensitive to is unknown."
It is important to point out that various un-optimized mixed cell-aggregation assays have successfully observed aggregation between N-cadherins (~20 uM, Linking molecular affinity and cellular specificity in cadherin-mediated adhesion), neurexin-3/neuroligin-1 (0.7-1uM, Koehnke 2010) and neurexin-1/LRRTM2 (20nM), which highlights the wide range of *in vitro* determined binding affinities of receptor/ligand pairs that sill aggregate in similar assays.

Figure 2: panel labels are swapped in A and C. In panel A, upper row is LRRTM2 showing aggregation with neurexin-3a SS4-; lower row is GFP. In panel C, the labels on the x-axis should read SS4- instead of SS4+.
We thank the reviewer for pointing out these mistakes and we have now fixed them on the figure.

In addition, I noticed several typos throughout the text. Please check carefully and clarify the following:

P2 line 28: 'interphases' - 'cellular interfaces' is probably meant here
The reviewer is correct, and we have made the appropriate spelling change.

P3 line 47: 'partner' - 'partners'
We thank the reviewer for catching this, and we apologize for the oversight. We have now pluralized the word.

P3 line 70 and 72: somewhat contradictory; interactions are usually observable in tens of minutes vs minutes later. Please clarify.
We thank the reviewer for their comment, and we have clarified in the text that it is "tens of minutes": "This aggregation is mediated exclusively by *trans* interactions and is usually observable in tens of minutes. No protein purification steps are required in this method, and the efficiency of the method relies on the paradigm that populations of HEK cells expressing cognate adhesion molecules are being combined and then imaged only tens of minutes later."

P3 line 86: LRRTM2 participates in synapse formation and maintenance - here it would be better to cite the three papers that showed this instead of just one: De Wit et al., Neuron 2009; Ko et al., Neuron 2009; Linhoff et al., Neuron 2009.
We thank the reviewer for seeing this and all three papers have now been be cited.

P4 line 91: instead of reference #10 (Linhoff et al., Neuron 2009), Siddiqui et al., J Neurosci 2010 should be cited here.
The citation has now been changed to 'Siddiqui et al., J Neurosci 2010'.

P4 line 100: 'Dublecco's'- 'Dulbecco's'
We thank the reviewer for alerting us of this mistake. The spelling has now been changed to 'Dulbecco's'.

P6 line 192: it would be helpful if it is clearly stated that the image at 60 minutes is designated 'time 60 image' in the rest of the text
We thank the reviewer for helping us make the manuscript more cohesive and we agree with their comment. We have thus added the following to the text: "Acquire final images at 60 minutes from now on designated as the 'time 60' image." Additionally, this comment helped us identify that we needed to do the same for 'time zero" images: "Assess baseline aggregation immediately after mixing the two conditions of HEK cells in step 1.8. These are now the 'time zero' images."

P6 line 194 and p8 line 284: 'minuets' - 'minutes'
We thank the reviewer for noticing these two spelling errors. They have now been changed.

P9 line 335: 'measure' - 'measured'.
The 'd' has been added to the end of 'measure' and the manuscript has been reviewed again in order to rid the text of other spelling and grammar errors.

1. Yamagata, A. *et al.* Structural insights into modulation and selectivity of transsynaptic neurexin–LRRTM interaction. *Nature Communications*. **9** (1), 3964, doi: 10.1038/s41467-018-06333-8 (2018).
2. Restrepo, S., Langer, N.J., Nelson, K.A., Aoto, J. Modeling a Neurexin-3α Human Mutation in Mouse Neurons Identifies a Novel Role in the Regulation of Transsynaptic Signaling and Neurotransmitter Release at Excitatory Synapses. *The Journal of Neuroscience*. **39** (46), 9065–9082, doi: 10.1523/JNEUROSCI.1261-19.2019 (2019).
3. Siddiqui, T.J., Pancaroglu, R., Kang, Y., Rooyakkers, A., Craig, A.M. LRRTMs and Neuroligins Bind Neurexins with a Differential Code to Cooperate in Glutamate Synapse Development. *Journal of Neuroscience*. **30** (22), 7495–7506, doi: 10.1523/JNEUROSCI.0470-10.2010 (2010).
4. Rebay, I., Fleming, R.J., Fehon, R.G., Cherbas, L., Cherbas, P., Artavanis-Tsakonas, S. Specific EGF repeats of Notch mediate interactions with Delta and serrate: Implications for notch as a multifunctional receptor. *Cell*. **67** (4), 687–699, doi: 10.1016/0092-8674(91)90064-6 (1991).
5. Fehon, R.G. *et al.* Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila. *Cell*. **61** (3), 523–534, doi: 10.1016/0092-8674(90)90534-L (1990).

6. Keith, F.J., Gay, N.J. The Drosophila membrane receptor Toll can function to promote cellular adhesion. *The EMBO journal*. **9** (13), 4299–4306 (1990).
7. Pandey, A., Jafar-Nejad, H. Cell Aggregation Assays to Evaluate the Binding of the Drosophila Notch with Trans-Ligands and its Inhibition by Cis-Ligands. *Journal of Visualized Experiments: JoVE*. (131), doi: 10.3791/56919 (2018).
8. Neupert, C. *et al.* Regulated Dynamic Trafficking of Neurexins Inside and Outside of Synaptic Terminals. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*. **35** (40), 13629–13647, doi: 10.1523/JNEUROSCI.4041-14.2015 (2015).
9. Ko, J., Fuccillo, M.V., Malenka, R.C., Südhof, T.C. LRRTM2 Functions as a Neurexin Ligand in Promoting Excitatory Synapse Formation. *Neuron*. **64** (6), 791–798, doi: 10.1016/j.neuron.2009.12.012 (2009).
10. Missler, M., Südhof, T.C., Biederer, T. Synaptic cell adhesion. *Cold Spring Harbor Perspectives in Biology*. **4** (4), a005694, doi: 10.1101/cshperspect.a005694 (2012).

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