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Detailed protocol

GPCR deorphanization assay in HEK-293 cells

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1.1 Introduction

Cell-surface receptors are membrane proteins that bind specific ligands, triggering cellular responses to regulate cellular functions. Orphan receptors, whose endogenous ligands are unknown, present unique challenges and opportunities in biology. Identifying ligands for these receptors – a process called deorphanisation – is crucial to uncover their physiological functions and to understand how cells communicate, process information, and adapt to changes. The technique described here was mostly used by us to identify ligands for neuropeptides. However, it has been used in the past by different groups for different ligands, including catecholamines, indolamines, GABA and other small molecules. Receptor deorphanisation is an important approach in the field of signalling and neurobiology, aiming to elucidate the intricate signalling mechanisms between and within cells.

This protocol was used in: Large-scale deorphanization of *Nematostella vectensis* neuropeptide GPCRs supports the independent expansion of bilaterian and cnidarian peptidergic systems. **doi**: https://doi.org/10.7554/eLife.90674.1

Keywords: Receptor, deorphanisation, ligand

1.2 Materials and Reagents:

1. Fetal Bovine Serum (FBS, heat-inactivated, we typically use **Gibco Cat. No. 16140071**)
2. Dulbecco's Modified Eagle's Medium (DMEM), while many brands could work, we used **Gibco Cat. No. 10569010**, which contains:

- High glucose (4.5 g/l)
- Supplemented with L-glutamine (alternatively, “Glutamax” for more stable glutamine)
- With Sodium pyruvate (110 mg/l)
- Optionally with Phenol Red indicator
- For culture, supplement this DMEM with 10% FBS

1. Opti-MEM (can include Glutamax, though it's not required, and should not contain phenol red, we used **Gibco Cat. No. 11058021**):
2. Dissociation solution: TrypLE or trypsin-EDTA solution (0.05-0.25% in HBSS or similar). We used **Gibco Cat. No. 12605010**.
3. Transfectamine 5000 (**AAT-Bioquest Cat. No. 60020**) or Branched Polyethylenimine with a molecular weight of 25k (PEI) diluted to 1 mg/ml (we used the brand: **Sigma-Aldrich Cat. No.9002-98-6**). The PEI option is extremely cheap when compared to any other transfection reagent available in the market. Thus, suitable for large-scale analyses.
4. Phosphate-Buffered Saline (sterile, we used **Gibco Cat. No. 70011044**)
5. Coelenterazine h (we use **PROMEGA Cat. No. S2011**, dissolve in Ethanol for a 2 mM stock: add 307 µl Ethanol to 250 µg Coelenterazine h)
6. HEK-293-G5a (Angio-proteomie **Cat. No. cAP-0200GFP-AEQ-Cyto**, HEK cells stably expressing the G5a-aequorin protein). Alternative, wild-type HEK-293 cells can be used.
7. Cell-freezing medium (we typically use **Gibco Cat. No. 12648010**).
8. Freezing containers (we use the **Corning® CoolCell™ SV2 Freezing Container. Cat No. CLS432010**).
9. FlexStation 3 Multi-Mode Microplate Reader.

1.3 Consumables

1. T25 (25 cm2) or T75 (75 cm2) cell culture flasks
2. 15 ml Falcon tubes
3. Sterile pipette tips
4. Sterile pasteur pipettes 3 ml

5. Sterile serological pipettes (10 ml & 25 ml)
6. White, clear bottom 96 well plates with lid, cell culture treated

1.4 Cell Culture general comments:

1. HEK-293 cells are cultured in DMEM (containing L-glutamine, sodium pyruvate, and high-glucose, and supplemented with 10% FBS) in a CO_2 incubator. This is known as working medium, or whole medium.
2. The cells' population doubles approximately every 1.5 days.
3. In our protocol, cell density is estimated visually, and not through cell counting.
4. Cells are centrifuged at 300 rcf for approximately 4 minutes in two 15 ml falcon tubes filled with 5 ml of cell-containing dissociating reagent and medium (TrypLE + DMEM).
5. Both the medium and the dissociation solution should be pre-warmed to 37°C before usage.
6. Regularly test the cells for mycoplasma contamination by PCR.

1.4.1 Dissociation and Resuspension of Cells:

1. For cells grown in a flask, the removal of the culture medium is the first step.
2. Optionally, cells can be rinsed with PBS.
3. Add 1.5 ml of TrypLE to a T25 flask, or 3.5-4 ml to a T75 flask.
4. Wait for five minutes and observe. For faster dissociation, flasks can be placed at 37°C. The cell layer should ideally break down into single cells without being over-digested. If cells are not dissociating well and tissue-like layers are floating around they can be gently mixed with a pipette.
5. Once cells have dissociated satisfactorily, add 5-10 ml of DMEM (supplemented with 10% FBS) with a serological pipette to stop the dissociation.
6. Spin the cells down in two 15 ml Falcon tubes, then discard the medium.
7. Add fresh medium and gently resuspend the cell pellet, we do this with a disposable, sterile Pasteur pipette. These cells can be frozen or used to set up new flasks or assays. This is explained below.

1.4.2 General comments about freezing and thawing of cells:

1. Cells should be frozen slowly (in the freezing containers) and thawed quickly (@ 37°C).
2. Only freeze cells that are in good condition, meaning they should not come from overgrown plates and should not have been cultured for extended periods without a medium change. How much the cells are diluted depends on the situation. It makes sense to prepare at least 1-2 new vials for freezing from the first passage after thawing. This is to maintain a healthy stock of frozen cells in early passages.
3. Before starting the protocol, we prepare several "ready to use" frozen vials, which will allow the deorphanisation process to be completed in one week. For this, we grow large amounts of cells in multiple T75 flasks. Divide each flask into two vials for freezing. When cells are healthy, one of these vials can usually be thawed on a Thursday evening or Friday morning, split into two T75 flasks and these flasks will be confluent on Monday. One can also divide a T75 flask into 4 vials and use the content for a single T75 flask when thawing. When preparing these vials, it is better to mix the cells of all T75 flasks that are grown simultaneously and then divide accordingly into freezing vials – this way all vials are equally healthy and the time that each vial of the batch needs to grow after being thawed can be determined.

1.6 Cell culture protocol

1.6.1 Freezing cells for assay or for long-term storage:

1. Grow flasks of cells until they are 90% confluent.
2. After cells are dissociated and centrifuged into a pellet (see “Dissociating Cells”), add the appropriate amount of freezing medium to the pellet instead of the culture medium. The freezing medium can be at room temperature.
3. Add cells into a cryovial and put them into the freezing container. Then, place them into a -70°C or -80°C freezer overnight. Freezing containers are designed so the temperature is lowered by about 1°C per minute to slowly freeze the cells.
4. Place the cells into a -150°C freezer or liquid nitrogen on the next day for long-term storage.

1.6.2 Culturing the cells:

1. Thaw a vial of cells at 37°C in a water bath.
2. Either spin down the cells in the freezing vial and remove supernatant directly, or add the entire content into a falcon tube containing DMEM and spin them down to a pellet to wash off more of the freezing medium.
3. Resuspend the pellet in warm DMEM (+ 10% FBS).
4. Fill a culture flask with fresh, warm DMEM (+10% FBS): ~8-10 ml for a T25 flask, or ~20-25 ml for a T75 flask.
5. Divide the resuspended pellet (after dissociation from a previous flask or after thawing) accordingly to reach the desired density. (Healthy HEK-293 cells need about 1.5 days to double.)
6. Use cells for the next passage, an assay or freezing latest when the flask is grown to ~95% confluency, so cells are only grown as a monolayer and not on top of each other. If overgrown, it will become difficult to estimate how many cells are there and cells will be less healthy.
7. When cells are seeded with a very low density or they don’t grow as fast as expected, the medium may have to be changed after a few days. Using a medium with phenol red as an indicator helps to tell when the medium is exhausted. To change the medium, simply remove the old medium and add medium while the culture flask is upright, so the medium is not added on top of the cells, but on the side of the flask to not apply a strong force onto the cell layer.

1.7 Deorphanisation assay protocol

1.7.1 Prepare cells for transfection:

1. Grow cells to ~95-100% confluency in a T25 or in a T75 flask.
2. Dissociate cells, spin down the pellet and resuspend the pellet in 5 ml fresh, warm DMEM (+10% FBS) per 96 well plates. A T25 flask of cells can be used to seed a single 96-well plate, which will require further addition of DMEM to ~10.5 ml, or a T75 flask can be used to seed three 96-well plates, in which case a total of ~31.5 ml will be needed.
3. Transfer 100 µl of Cell suspension into each well. Keep the cells suspended during this process, so each well gets the same amount of cells (pipette up and down every time when going into the suspension). It is best to use a pipetting reservoir for this and an 8-channel or 12-channel pipette.

4. Cultivate cells in a 96-well plate for a period of two days, aiming for approximately 90% confluency. Monitor the cells routinely to avoid overgrowth, which could hamper the effectiveness of transfection. Simultaneously, ensure that they are not undergrown, since the transfection solution can be toxic to insufficiently proliferated cells.

1.7.2 Transfection:

1. Prepare the transfection mix: per well, mix 10 µl OptiMEM (without FBS) + 60-70 ng GPCR plasmid + 60-70 ng Gq protein plasmid + 60-70 ng G5A plasmid, if normal HEK cells are used (unless a line with stable G5A expression is used) + 0.3 µl PEI or Transfectamine 5000 per 100 ng total DNA. Mix and incubate for 20 minutes.

Either:
Exchange medium in the 96 well plates with 90 µl of (warm) OptiMEM (supplemented with 5% FBS). Be careful when taking out the medium so as not to disturb the cell layer – lower the pipette towards one side of each well. When adding the fresh medium, best add medium against the opposite wall of the well. Add 10 µl of the transfection mix per well. This method is typically used when testing different receptors in each row of the plate.
Or:
When a whole (or half) plate is transfected with the same receptor construct, it is easier to incubate the transfection mix for 20 minutes and then mix it with the fresh OptiMEM (5% FBS) and then exchange the medium of the plate with the Transfection mix/OptiMEM mixture. Calculate accordingly for some dead volume and add in the same ratio as otherwise (90 µl OptiMEM + 10 µl transfection mix per well). Let the cells express the proteins for 2 days. The transfection mix is slightly toxic for the cells and the DNA prep may also contain endotoxin carryovers. Best results seem to be obtained when the transfection mix is incubated for 4 hours with the cells and then replaced by fresh OptiMEM (5% FBS). If cells are healthy, it also works to keep the mix on the cells until the assay or exchange it with fresh OptiMEM (5% FBS) the day after transfection.

1.7.3 Cell Assay:

1. Two days post-transfection, replace the medium with 50 µl per well of fresh OptiMEM, WITHOUT phenol red and WITHOUT FBS, but supplemented with 4 µM Coelenterazine h (add 2 µl of a 2 mM Coelenterazine h stock per ml of medium).
2. Keep the plate in the dark (we cover them with tin foil or keep them inside cardboard boxes) and incubate for 2 hours at 37°C. Cells can be incubated in a normal incubator without CO_2.
3. Prepare the ligand plate by dissolving ligands in DMEM or OptiMEM without FBS. 0.1% sterile filtered BSA can be added. Remember to adjust the concentration in the ligand plate such that the final concentration during the assay is known. Usually, we add 50 µl of ligand to 50 µl of cell medium, so we prepare the ligand plate with double the concentration of the final intended measurement concentration. Calculate for some dead volume per well (we usually added 25 µl per well more than actually used for the assay). When using a gradient within a row, best is to start on the left side with low concentrations and go towards the right side for higher ligand concentrations (this way the same tips can be used in the machine for the entire row as the machine injects from left to right). We usually use a 10 µM concentration of ATP in the last column as a general control to test if the cells are healthy enough and emit sufficient luminescence for the assay.

1.8 Reading the plate:
The machine has different protocols ready for measurement. There is a specific one for aequorin-based GPCR assays. Open the software max 7.1 software and go to:
Protocol manager
-Protocol library
- Cell signalling and transport.
-Aequorin (This should open a blank template)

Choose the template for 96 well plates and press settings (the double cogs symbol in yellow). Pressing this button will give you a list of setups to modify in your plate reading. Here there is the list of setups to do.

1. **Wavelengths**: Make sure the option of all is ticked.
2. **Plate type:** Choose plate type, this depends on the brand you are using in our case we used COSTAR white plate/clear bottom.
3. **Read area:** Choose the area to read.
4. **PMT and optics. Integration**: this depends on how many rows you are reading, if you read 4 rows, 150 or 100 msec will work. If reading the 8 rows, 75 to 100 milliseconds will allow the reading of the whole plate while minimising the loss of the coelenterazine signal (avoid using higher integration times when reading many rows).
5. **Timing:** Choose the minimum time possible by the machine, this time will depend on the integration time chosen and the number of rows being read.
6. **More settings:** Make sure calibration is ticked
7. **Compound transfer:** Here, choose the volume to be injected, the speed and the time in which you want the injection to happen. In our case, we add 50 microliters of peptide solution. Since the cells are in 50 µl of medium, we can calculate the final molarity in each case. If you change this, consider the final concentrations.
    1. Pipette height 50 µl
    2. Volume 50 µl
    3. Rate 2 (speed of injection, this is kept slow to avoid the speed of the injection displacing the cells)
    4. Time point. This is the time of injection after the baseline readings. Use from 16 to 18 seconds.
8. **Compound source:** choose the type of compound plate you are using. We typically use Costar flat bottom 0.3 ml.
9. **Compound and tip columns:** Here you have to choose which tip column is using which column of the compound plate and in which column of the assay plate it is going to be injected. Typically, tip 1 takes from a compound plate well 1 and injects in plate well 1. However, this can be changed, for example, when making replicates. Tips 1-6 can be used to read the whole plate, saving half of the tips. The machine will read from the left side of the plate towards the right side, which is to be kept in mind when re-using the same tips at different concentrations.
10. **Triturate:** This can be used to mix the compound once added. This is not good for cell assays, and they may detach, die and potentially produce false positives, we keep it off.

1.9 Data analysis

1. Save the plate readout separately and then export the data as ".xls" file in the "reduced" format.

2. You can analyse the data with the R script "Dose_response_curves.R" which is provided in the GitHub repository https://github.com/JekelyLab/Thiel_Yanez_Nematostella (in the "code" folder)
3. Transformed the data to a ".csv" format with the different concentrations in different columns and the different peptide-receptor pairs and replicates in different rows. The script will read columns labelled as followed: Replicate, Receptor, Peptide, 0, 1.00E-13, 1.00E-12, 1.00E-11, 1.00E-10, 1.00E-09, 1.00E-08, 1.00E-07, 1.00E-06, 1.00E-05, 1.00E-04.
4. Name the columns in the datafile in the same way and number the different replicates of each peptide-receptor pair. Empty data points are not a problem and will be ignored (for example if one only measures concentrations from 1E-11 to 1E-05 instead of 1E-13 to 1E-04). The file "Nvec_Dose_response_assays.csv" on the github repository (in the "data" folder) can be used as an example for how to arrange the data.
5. The readout data are first converted to tibble format and then fitted with the drc package to calculate EC_{50} values.

How to cite:

1. Thiel, D. , Yanez Guerra, L. A. and Jékely, G. (2023). GPCR deorphanization assay in HEK-293 cells. Bio-protocol Preprint. 10.21769/p2493.