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TITLE:
Construction of Model Lipid Membranes Incorporating G-protein Coupled Receptors (GPCRs)

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SUMMARY:
This protocol utilizes agarose swelling as a powerful and generalizable technique for incorporating integral membrane proteins (IMPs) into giant unilamellar lipid vesicles (GUVs), as described here for the reconstitution of the human 1A serotonin receptor protein (5-HT_{1A}R), one of the classes of pharmacologically important G protein-coupled receptors.

ABSTRACT:
Robust *in vitro* investigations of the structure and function of integral membrane proteins has been a challenge due to the complexities of the plasma membrane and the numerous factors that influence protein behavior in live cells. Giant unilamellar vesicles (GUVs) are a biomimetic and highly tunable *in vitro* model system for investigating protein-membrane interactions and probing protein behavior in a precise, stimulus-dependent manner. In this protocol, we present an inexpensive and effective method for fabricating GUVs with the human serotonin 1A receptor (5-HT_{1A}R) stably integrated in the membrane. We fabricate GUVs using a modified hydrogel swelling method; by depositing a lipid film on top of a mixture of agarose and 5-HT_{1A}R and then hydrating the entire system, vesicles can be formed with properly oriented and functional 5-HT_{1A}R incorporated into the membrane. These GUVs can then be used to examine protein-membrane interactions and localization behavior via microscopy. Ultimately, this protocol can advance our understanding of the functionality of integral membrane proteins, providing profound physiological insight.

INTRODUCTION:
Synthetic model membranes are powerful tools in the investigation of the fundamental properties and functions of biomembranes. Giant unilamellar vesicles (GUVs) are one of the most prominent platforms to study a variety of plasma membrane properties and can be

engineered to mimic different physiological conditions[1–8]. It is well established that the plasma membrane and its organization play a key role in a multitude of cellular processes, such as signal transduction, adhesion, endocytosis, and transport[9–15].

GUVs have been fabricated using various methods, including gentle hydration[16], hydrogel swelling[17], electroformation[18], microfluidic techniques[19–22], jetting[23], and solvent exchange[24–26]. Due to challenges in handling integral membrane proteins (IMPs), *in vitro* platforms to study them have been limited. GUVs present a simplified platform for studying IMPs in an environment that mimics their native environment. Although there have been several approaches for protein reconstitution in GUVs, challenges arise from incorporating proteins with the correct orientation and maintaining protein functionality[27].

Most successful protein-reconstitution in GUVs requires the detergent exchange method; which involves solubilizing the proteins from their native environment by detergents, followed by protein purification, and then replacing the detergent molecules with lipids through various methods[28]. While detergents serve to stabilize the tertiary structure of IMPs during purification, detergent micelles are a relatively unnatural environment for these proteins, which are better stabilized, particularly for functional studies, in lipid bilayers[28–30]. Moreover, incorporating functional transmembrane proteins into the lipid bilayer using traditional GUV fabrication techniques has been difficult due to the size, the delicacy of these proteins, and the additional detergent exchange steps that would be needed[27,31–33]. The use of organic solvent to remove detergents causes protein aggregation and denaturing[34]. An improved detergent-mediated method has been promising, however, caution is needed for the detergent removal step and optimization might be needed for specific proteins[31,35]. Additionally, methods that utilize electroformation could restrict the choice of protein and may not be suitable for all lipid compositions especially charged lipids[31,36,37]. Another technique that has been used is peptide-induced fusion of large unilamellar vesicles (LUVs) containing the desired protein with GUVs, though it was found to be laborious and can lead to the insertion of foreign molecules—the fusogenic peptides[33,38,39]. Giant plasma membrane vesicles (GPMVs), which are derived from living cells, can be used to overcome some of these issues, however they allow minimal control of the resultant lipid and protein composition[14,40,41]. Therefore, the integration of IMPs in the bilipid layer of GUVs using our modified agarose swelling method presents a reliable method to further examine these proteins in the membrane environment[42–45].

Cellular signaling and communication involves a family of proteins known as G protein-coupled receptors (GPCRs); GPCRs are among the largest family of proteins and are associated with modulating mood, appetite, blood pressure, cardiovascular function, respiration, and sleep among many other physiological functions[46]. In this study, we used human serotonin 1A receptor (5-HT_{1A}R) which is a prototypical member of the GPCR family. 5-HT_{1A}R can be found in the central nervous system (CNS) and blood vessels; it influences numerous functions such as cardiovascular, gastrointestinal, endocrine functions, as well as participating in the regulation of mood[47]. A large barrier to GPCR research arises from their complex amphiphilic structure, and GUVs present a promising platform for the investigation of various properties of interest, ranging from protein functionality, lipid-protein interactions, and protein-protein interactions.

Various approaches have been utilized to study lipid-protein interactions such as surface plasmon resonance (SPR)[48,49], nuclear magnetic resonance spectroscopy (NMR)[50,51], protein lipid overlay (PLO) assay[51–54], native mass spectrometry[55], isothermal titration calorimetry (ITC)[56,57], and liposome sedimentation assay[58,59]. Our lab has used the simplified GUV approach to investigate the effect of lipid-protein interactions on protein functionality by incapsulating BODIPY-GTPγS, which binds with the $G_{i\alpha}$ subunit in the active state of the receptor. Their binding unquenches the fluorophore producing a fluorescence signal that could be detected over time[45]. Moreover, various studies investigated Lipid-protein interactions and the role of proteins in sensing or stabilizing membrane curvature[60,61], and utilizing a feasible GUV approach could be a key advantage.

This protocol demonstrates a straightforward method to incorporate GPCRs into the membrane of GUVs using a modified agarose hydrogel system[17,42]. Furthermore, based on our previous work, our method could be suitable for IMPs that can bear short-term exposure to 30–40 °C. Briefly, we spread a thin film of agarose combined with membrane fragments containing the GPCR of interest. Following gelation of this layer, we deposit a lipid solution atop the agarose and allow the solvent to evaporate. Rehydration of the system was then performed with an aqueous buffer, resulting in the formation of GUVs with protein incorporated in the lipid bilayer.

PROTOCOL:

1. Protein labeling

1.1. Allow NHS-Rhodamine, 5-HT_{1A} membrane fragments, and one 7 K MWCO spin desalting column to equilibrate at room temperature.

1.2. Dissolve 1 mg of NHS-rhodamine in 100 µL of dimethyl sulfoxide (DMSO).

1.3. Add 5 µL of 1 M sodium bicarbonate solution to increase the pH of 5-HT_{1A}R solution to pH 8.

1.4. Add 3.66 µL of the NHS-rhodamine solution to 50 µL of the 5-HT_{1A}R solution and pipette gently up and down in a microcentrifuge tube.

NOTE: Ensure to have at least 10x molar excess of NHS-rhodamine.

1.5. Keep the mixture protected from light and put on rotator at room temperature for 1 h.

1.6. Wash a 7 k MWCO spin column with 200 µL of 1x phosphate buffer saline (1x PBS) three times for 1.5 min at 1.5 RCF for each wash.

1.7. Add the labeled protein to one column and balance the amount in another microcentrifuge tube.

1.8. Spin down the labeled protein once for 5 min at 1.5 RCF.

1.9. Take a UV-vis spectrum using a nanodrop spectrophotometer at 280 nm and 554 nm and calculate the labeling efficiency following the manufacturer's manual.

1.10 Store the labeled protein covered at 5 °C until further use. The solution is stable for approximately a week after labeling.

2. GUVs with membrane-incorporated 5-HT_{1A}

2.1. Preparation of materials and reagents

2.1.1. Allow the protein, lipids and BSA (Bovine serum albumin) to equilibrate to room temperature.

2.1.2. During this time, clean the coverslips by placing them in methanol and sonicating for 30 min at 40 °C. Ensure that the methanol completely covers the coverslips and the water level in the water bath is above the level of the methanol in the container.

NOTE: Methanol is toxic and should be handled in appropriate chemical hood.

2.1.3. Dry off the excess methanol on the coverslips with a gentle stream of air. Place the coverslip rack covered in a 40 °C oven for 15 min to ensure that the excess coverslips dry off.

2.1.4. Begin the plasma cleaning process. First, place the coverslips into the plasma cleaner and close off the air intake valve to evacuate all the air inside the chamber.

2.1.5. Once the chamber is under vacuum, clean the coverslips for 5 min using high RF power setting and a near complete vacuum, with only a slight air intake into the vacuum chamber. To ensure the proper level of plasma, adjust the opening of the vacuum chamber such that the resultant color of the plasma is a steady, bright pink.

NOTE: It is crucial when using air that the plasma remains a bright pink color for the duration of the plasma treatment step, as a darker purple color indicates that there is an improper amount of air in the chamber and will result in a suboptimal plasma treatment.

2.1.6. Once the 5 min have passed, shut off the RF power and release the vacuum.

NOTE: Upon removal from the plasma chamber, please ensure that the coverslips remain covered.

2.2. Hydrogel preparation

2.2.1. Combine 6 mg of ultra-low melting temperature agarose with 300 µL of ultrapure water (i.e., 2% (w/v) agarose).

NOTE: 2% agarose will be used to make protein-free GUVs. Agarose solution can be kept at 45 °C for two days.

2.2.2. Combine 9 mg of ultra-low temperature agarose with 300 µL of ultrapure water for 3 w/v% agarose by as prepared in step 3.1. 3% agarose will be used to make protein incorporated GUVs.

2.2.3. Vortex the solution briefly before placing them on the 90 °C heat block for 10 min. Then, vortex the tube again before transferring it to a 45 °C heat block to keep it in the molten form until further use.

2.3. **Agarose and protein mixing**

2.3.1. Mix 21 µL of 3% agarose with the 7 µL of 5-HT_{1A}R membrane fragments. Pipette up and down slowly many times to ensure adequate mixing. Then, incubate at 45 °C for 1 min.

2.4. **Hydrogel and lipid deposition**

2.4.1. For protein-free GUVs: Make a thin film on freshly plasma-cleaned coverslips using 20 µL of 2% agarose. Quickly drop another coverslip on top of the agarose droplet and gently slide the coverslips apart to make a thin film on both coverslips.

NOTE: This step is tricky in that the sliding of the droplet must occur while the agarose is still in the molten form.

2.4.2. For protein-incorporated GUVs: Pipette the protein/agarose mixture up and down one more time, and then deposit 20 µL of the 2% agarose on a plasma-cleaned coverslip. Follow the slip-casting directions as described above.

2.4.3. Allow the agarose to gel protected from light for 30 min at room temperature.

2.4.4. Deposit the lipids dropwise on top of the agarose layer. Use a total of 10 µL of 2 mg/mL of 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) with 0.4 Mol% 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) labeled with ATTO 488 (ATTO-488-DPPE) (or lipid mixture of interest) in chloroform on top of the agarose film. Deposit the droplets using a gas chromatography needle and spread one droplet at a time around via a gentle air stream.

NOTE: Caution is needed with this step to make a relatively uniform layer of lipids on top of the hydrogel. Also, chloroform is toxic and should be handled in appropriate chemical hood.

2.4.5. Assemble the Sykes-Moore (S-M) chambers by placing an O-ring on top of the coverslip, and then placing the top component of the chamber on top of the O-ring. Use the key provided by the manufacturer to assemble the chamber by screwing the chamber components together to seal the chamber and prevent any leakage.

NOTE: The top of the chamber should be tightened on the O-ring but caution is needed to ensure the coverslip stays intact as the coverslip can crack if the O-ring doesn't sit properly in the chamber. Also, ensure that the chamber is sealed tight enough such that the chamber does not leak when the swelling solution is added. Failure to tighten the chamber enough will result in leaks and loss of sample.

2.5. **Swelling and harvesting of vesicles**

2.5.1. Hydrate the entire system by gently pipetting 450 µL of 200 mM sucrose in 1x PBS and gently tapping the chambers to ensure adequate buffer coverage of the hydrogel-lipid layers.

NOTE: The sucrose solution can be replaced with a rehydration buffer containing biological probes of interest.

2.5.2. Place the chambers at 45 °C and cover the top part of the chamber with a coverslip to prevent evaporation. Allow the sample to swell, protected from debris and light for 1 h.

2.5.3. Add 100 µL of 1 mg/mL BSA in ultrapure water into each well of a 96-well plate intended to be used. Incubate at room temperature for 1 h.

2.5.1. Wash three times with ultra-pure water and once with 200 mM sucrose in 1x PBS.

2.5.2. Finally, add 200 mM of glucose in 1x PBS until the addition of the GUV sample solution.

NOTE: BSA was used to block GUV adsorption.

2.5.6. After allowing the hydrogel to swell, gently shake and tap the chamber to dislodge any GUVs that may remain attached to the hydrogel surface. Then, carefully pipette up the GUV-sucrose solution.

NOTE: As an optional step to ensure all vesicles are detached from the surface, gently pipette some of the sucrose suspension back onto the hydrogel surface.

2.5.7. Move the suspension into a previously prepared microcentrifuge tube containing 700 µL of 200 mM glucose in 1x PBS.

NOTE: The density gradient will lead to settling of the vesicles to the bottom of the centrifuge tube.

2.5.8. Allow the vesicles to settle for another hour to ensure that the vesicles can sink to the bottom of the microcentrifuge tube, allowing for optimal collection.

2.5.9. After the settling of GUVs in glucose, transfer 300 µL from the bottom of the centrifuge tube (the settled vesicles) into the previously prepared and BSA-treated 96-well plate to examine the vesicles under the confocal microscope.

NOTE: Be sure to avoid the very bottom of the microcentrifuge tube to minimize the amount of debris collected in the final sample.

2.6. **Check the samples under the microscope.**

2.6.1. Shine a 488 nm laser on the sample (that allows us to visualize the membrane, as the bilayer has been labeled with ATTO-488-DPPE).

2.6.2. Shine a 561 nm laser on the sample (that allows us to visualize the protein, since it has been labeled with NHS-Rhodamine).

NOTE: Caution is needed while imaging the sample as photooxidation can destabilize the vesicles. Vesicles were observed on the same day.

[Place **Figure 1** here].

REPRESENTATIVE RESULTS:

The concentration of protein was measured, and the degree of labeling was calculated as the molar ratio between the dye and the protein to be 1:1. By examining the GUVs using confocal microscopy, we were able to confirm successful formation and protein integration of the vesicles. The lipids were labeled with 0.4 mol% ATTO 488-DPPE, and the protein was covalently labeled via rhodamine NHS-ester modification of primary amines. **Figure 2a** and **Figure 2b** show a protein-incorporated vesicle in the ATTO 488 and rhodamine channels, respectively. All micrographs have been dark current and flatfield corrected. **Figure 2c** and **Figure 2d** show a negative control GUV with no protein incorporated. **Figure 3a** and **Figure 3b** show a protein incorporated GUV with line intensity profiles given by the dashed white line of the same vesicle in both channels. The line intensity profile shows a two-dimensional plot of the intensities of the pixels along the white drawn line within the image. The x-axis is the distance along the line and the y-axis is the pixel intensity. ImageJ software was used to plot the profile intensity of the indicated line.

[Place **Figure 2** here].

[Place **Figure 3** here].

FIGURE LEGENDS:

Figure 1: Illustration of the detailed protocol steps. Created with BioRender.com

Figure 2: Micrographs comparing protein incorporated GUVs and GUVs without protein (control). Micrographs (**a**) and (**b**) show protein incorporated GUV fluorescence with the respective ATTO 488 and rhodamine channels, respectively. Micrographs (**c**) and (**d**) show a protein omitted GUV when excited with ATTO 488 and rhodamine channels, respectively.

Figure 3: Top row shows micrographs of protein incorporated GUVs in ATTO 488 (a) and rhodamine (b) channels. Line intensity profiles for the indicated white-dashed lines are below. The analysis was performed using ImageJ software.

DISCUSSION:

We have identified two steps that are critical to the success of the overall protocol: plasma treatment and lipid deposition. Plasma cleaning of the coverslips is essential in ensuring that there is adequate coverage and adhesion of the agarose hydrogel to the glass coverslip. Plasma cleaning accomplishes two things: first, it removes traces of organic matter from the glass surface; second, it activates the coverslip surface, allowing for an increase in wettability as the glass surface hydrophilicity increases[62,63]. Touching the coverslip surface post-plasma cleaning will inactivate and contaminate the ultraclean surface and is strongly advised against. Our recommendation is to only touch the very edges and undersides of the coverslip when handling the coverslips for the agarose slip casting step. The second critical step is the deposition of lipids onto the dry hydrogel surface. This method uses a dropwise lipid deposition, which requires a gas chromatography (GC) needle and an air stream to deposit a few microliters of lipid solution at a time, allowing for precise control of the amount of lipid added and the placement of the lipid film on the hydrogel surface. The drawback of this method is that if not done carefully, it can result in a few select areas with a thicker lipid film, resulting in reduced GUV yields. Thus, it is critical to ensure that there is as uniformly thin of a lipid layer as possible on the surface of the agarose.

One of the most significant benefits of this protocol is the flexibility of the platform itself; this method lends itself very well to changes in protein and lipid composition, as well as encapsulation and buffer modifications. This protocol can, in principle, include any transmembrane protein, as we have been able to successfully incorporate a number of different transmembrane proteins, ranging from the adenosine receptor ($A_{2A}R$) to plant aquaporins without sacrificing functionality[42,45,64]. Traditionally, proteins have been incorporated into GUVs following solubilization by detergents or incorporation into proteo-liposomes or small unilamellar vesicles that can be subsequently integrated into a preformed GUV[65]. The advantage of our modified hydrogel swelling method is that it removes the dependency of detergents or intermediate vesicles and provides an intermediate hydrated scaffold. The benefits of this are twofold: we can stably incorporate functional GPCRs into the membrane in a more physiologically relevant buffer without relying on detergent exchange methods that require more preparation and care regarding the concentration of the said detergents, and that the process by which GUVs bud off the surface of the hydrogel allows for the correct orientation of the proteins in the bilayer[66]. We have shown that the GUV budding process involves the coalescence of many smaller nanometer-scale vesicles into larger, micron-

scale vesicles, which encourages correct protein orientation from the beginning. We have shown this to be the case in our previous work; in short, we covalently labeled an antibody targeting a specific cytosolic loop of the Adenosine receptor and incubated the labeled antibody with the protein, and then incorporated the labeled protein into lipid-dye-labeled GUVs using the modified hydrogel swelling method. We then exposed the protein-incorporated vesicles to a charged quencher, which is unable to cross the bilayer. We subsequently see a 50% reduction in fluorescence of the lipid dye, but the fluorescence of the labeled protein remains unaffected by the quencher, demonstrating proper orientation[44].

Previous work out of our lab has investigated the role in which lipid headgroup charge, zwitterionic and net-ionic charged lipids, as well as buffer and hydrogel properties such as pH, ionic strength, osmolarity, and hydrogel concentration have on the dynamics of GUV formation[67]. In short, lipid charge does not largely affect GUV formation, while buffer properties such as increases in sucrose concentration (e.g., 500 mM Sucrose in 185 mM ionic strength PBS buffer) negatively affect GUV formation, resulting in irregularly shaped vesicles that most likely will not readily lend themselves to harvesting. Acidic solutions (pH = 3) increase the rate of formation, while a more basic solution (pH = 8) suppresses the rate of GUV formation. GUVs still form at both the acidic and basic buffers, with only marginal differences in vesicle size. Low agarose concentrations (~0.1–1 w/v%) also negatively affect GUV formation due to a lack of homogenous surface coverage and a decrease in hydrogel swelling, a necessary force in the coalescence and budding of GUVs off the hydrogel surface. Thus, we have determined that a 2 w/v% final agarose concentration with a sucrose/glucose solution of 100–200 mM, combined with a buffer ionic strength of 185 mM PBS at pH 7.4 achieves a good balance of agarose swelling, GUV formation rate, and subsequent vesicle size. For vesicles that contain protein, increasing the initial agarose concentration to 3 w/v% allows for a final agarose concentration of 2 w/v% after the addition of the protein solution. In addition to formation dynamics, the sucrose/glucose buffer system also facilitates the sedimentation and subsequent collection of formed GUVs, as well as visualization under phase contrast microscopy[65,68].

There are some points of caution regarding this protocol, specifically regarding the agarose and the selection of vesicles. For instance, while we use an ultralow melting temperature agarose, the agarose-water suspension needs to reach at least 60 °C to become molten, and the agarose-protein mixture is incubated at 45 °C. In our experience, this temperature does not eliminate the activity of 5-HT_{1A}R, but caution is warranted for other proteins. In general, the agarose we use begins to gel at 20 °C and thus the swelling reaction can take place at temperatures above 20 °C, but this process cannot function below that temperature. It should also be noted that the closer the temperature gets to 20 °C, the less efficient the swelling step becomes, leading to subsequent decreases in GUV yields. The agarose can also present an issue during the settling and visualization steps, as it can persist at the bottom of the settling/collection tube as debris. Thus, caution is required for the temperature required to maintain the molten agarose and ensuring that the said temperature will not denature the protein of interest as well as aspirating the settling solution to avoid any excess suspended agarose from being included in the final sample. This method in its current state also results in a heterogeneous GUV population size, with some vesicles displaying multilamellarity and other flawed vesicle

phenomena such as vesicles within vesicles. This is typical of common GUV formation methods and requires vigilance and discretion when selecting vesicles for microscopy and analysis. GUVs that display unusually high levels of fluorescence are also not recommended for analysis, as agarose can be found on the interior of some of these vesicles. Unpublished work out of our lab has been able to run micropipette aspiration experiments using vesicles made using this technique, illustrating that the agarose method produces vesicles without mechanics-altering agarose in the lumen.

Limitations aside, this protocol presents a robust and straightforward method for generating protein incorporated GUVs. It can generate high yields of GUVs in physiologically relevant conditions that incorporate properly oriented transmembrane proteins into the bilayer without compromising their functionality. This is a departure from other methods of vesicle formation, which involve electric currents or gentle hydration, that would significantly damage the structure of the protein and render it nonfunctional or require further detergent solubilization and removal steps. Given that GPCRs represent upwards of a third of all pharmaceutical targets, there is significant interest in being able to study this family of proteins in a highly tunable, high-throughput, biomimetic platform. More specifically, the applications of this work range from the study of protein-lipid interactions, how the lipid microenvironment influences protein functionality and localization, and other basic biophysical questions that can inform pharmaceutical drug development and discovery. An example of this can be found in the work completed within our lab, which has been able to discern variances in receptor functionality as a result of lipid oxidation.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure

Click here to access/download;Figure;Figure 1.tif

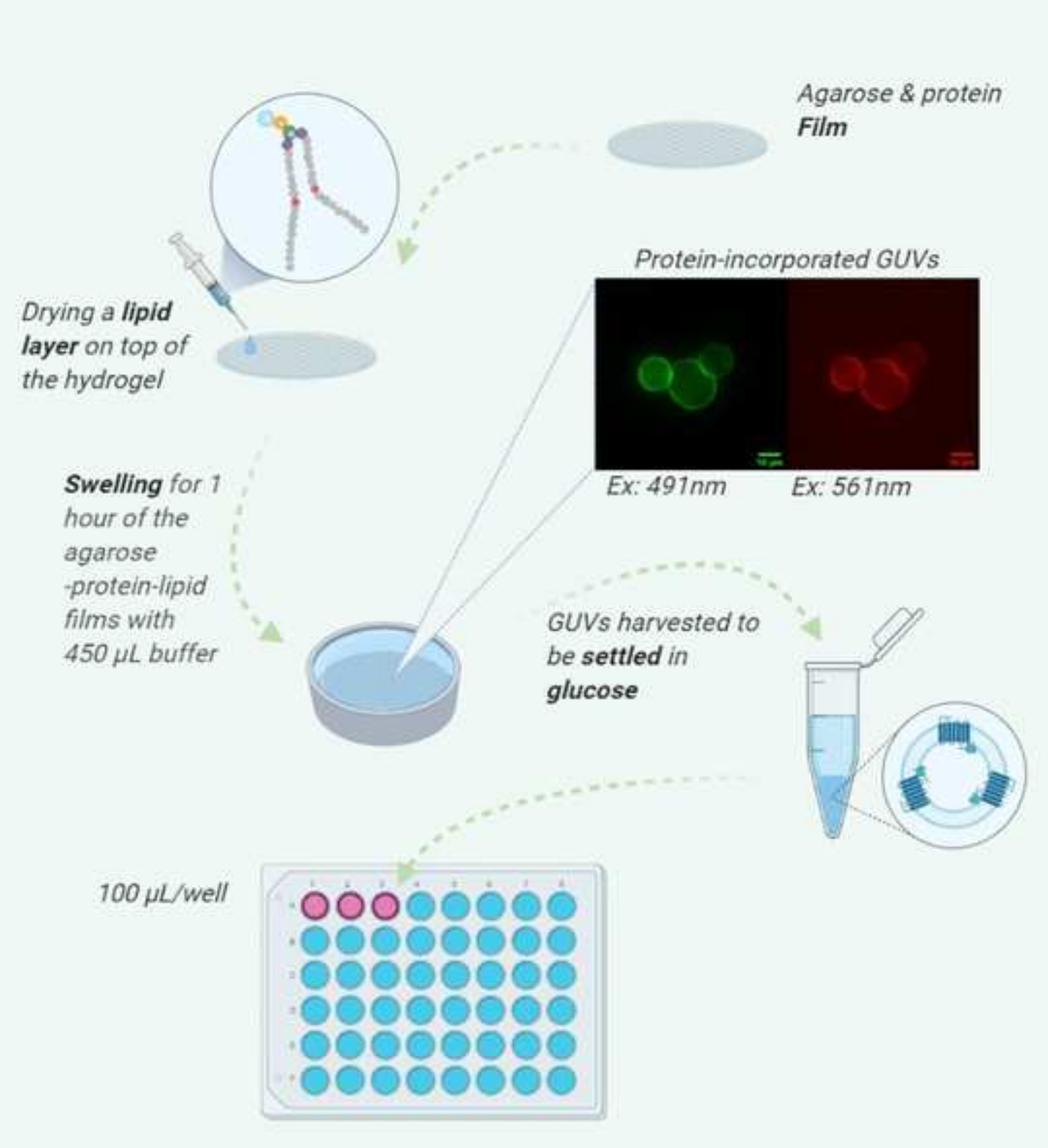

Figure 2

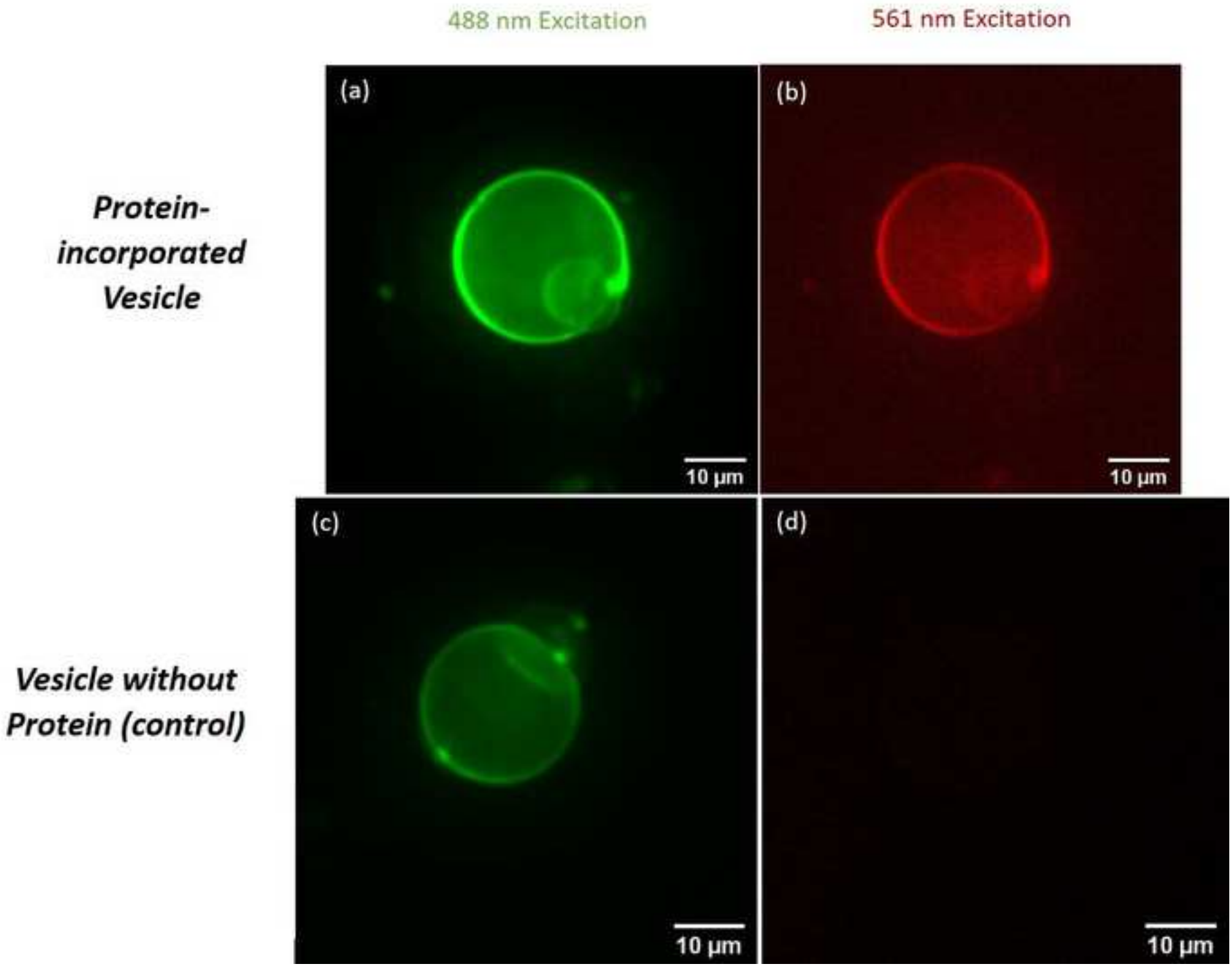

Figure 3

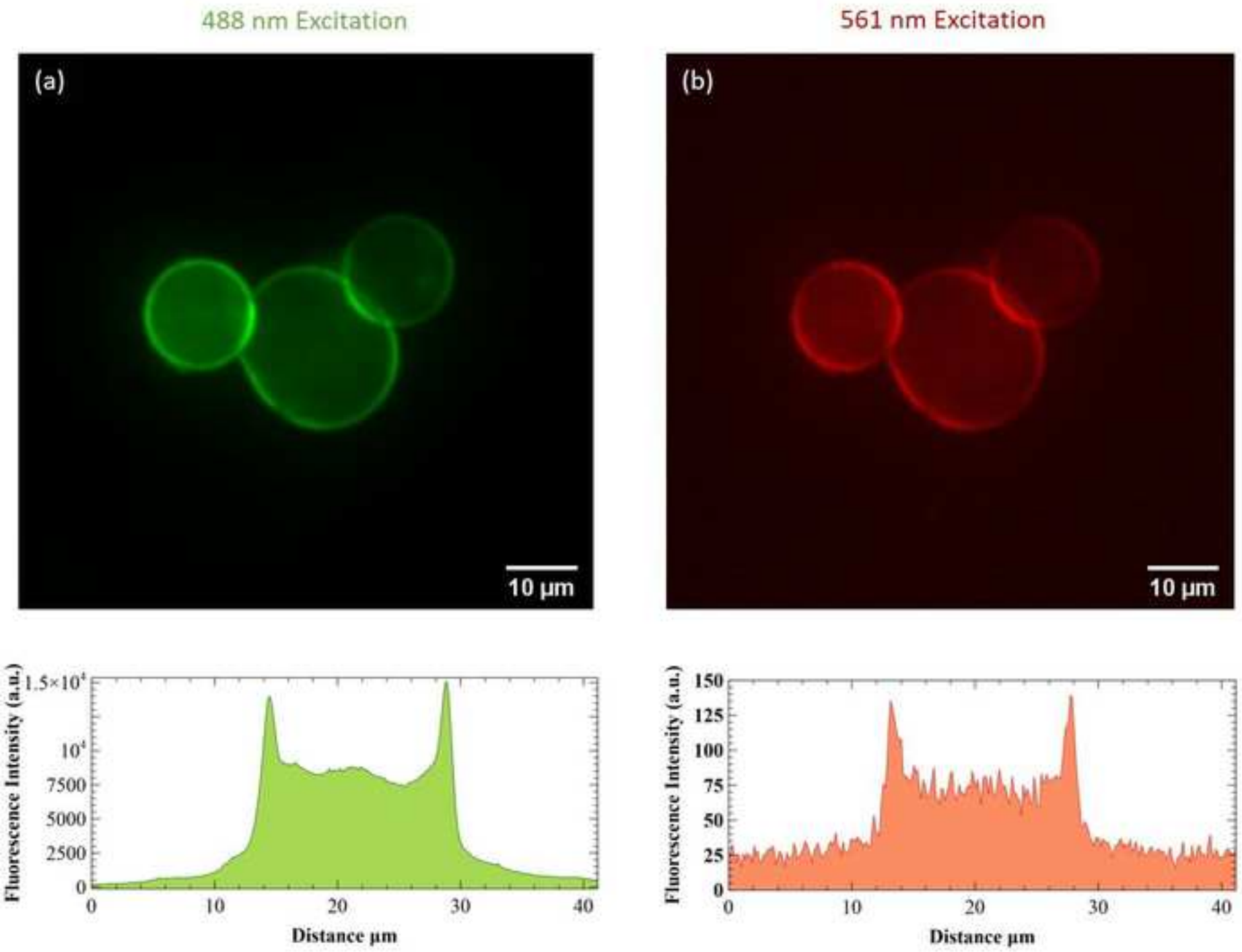

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Table of Materials

Materials.xlsx

Review Response to Construction of Model Lipid Membranes Incorporating G-protein Coupled Receptors (GPCRs)

Ahmed Elbaradei, Lucia C. Dalle Ore, Noah Malmstadt

Email: malmstad@usc.edu

Response to editorial staff:

We appreciate the opportunity to revise our work for submission to the Journal of Visualized Experiments. We are grateful for the time and energy that the editorial staff have dedicated for the feedback to our manuscript and video. In this response, editorial comments are in *blue text* and our response are in black text.

Changes to be made by the Author(s):

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

We have thoroughly checked the document for grammar and spelling errors.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

We have updated the manuscript as indicated.

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "This protocol presents…"

We have updated the Short Summary as indicated; see line 48.

4. Please ensure the Introduction include all of the following with citation:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

We have updated the manuscript as follows:

a) See text beginning on line 169: "This protocol demonstrates a straightforward method to incorporate GPCRs into the membrane of GUVs using a modified agarose hydrogel system.."
b) Lines 124-126 explain the rationale behind the utilization of giant unilamellar vesicles (GUVs) as a platform for investigating integral membrane proteins for their ability to mimic their native environment and study the effect of specific lipid structures and compositions in a reductive manner. Lines 129-149 lay out the shortcomings of traditional GUV fabrication methods in incorporating integral membrane proteins, providing a justification for using the technique described here.

c) Lines 124 and 125 discuss the advantage of our approach as an in vitro platform to study integral membrane proteins. Also, Lines 129-149 present different approaches and techniques and their disadvantages compared to the presented technique.
d) Line 169-176 briefly discuss the technique supported with previous literature. Also, Lines 129-149 discuss previous literature about giant unilamellar vesicles (GUVs) formation techniques and protein incorporation approaches.
e) Line 170 points out how the temporary exposure of proteins to 30-40 °C may restrict the application of the presented approach for some proteins.

5. Please move the discussion to the end after the figure legend section. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have updated the manuscript as follows:

a) The paragraph starting from Line 337 discusses in depth the critical steps involved in the protocol.
b) Lines 354-359 discuss possible modifications of the technique; Line 376 highlights troubleshooting parameters for GUV formation.
c) Lines 397-418 point out limitations of the protocol.
d) Lines 419-427 examine the significance of the protocol compared to other methods.
e) Line 427-432 propose potential applications and provide a previous example of research using GUVs incorporating IMPs.

6. Please cite the references in order.

We have updated the manuscript as suggested.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

We have updated the manuscript as indicated. We changed "would allow" to "allows" in lines 301 and 303.

8. Please ensure you answer the "how" question, i.e., how is the step performed?

We have revised the protocol to better reflect this recommendation.

9. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

We have updated the manuscript as indicated beginning at line 329.

10. Please ensure the results are described in the context of the presented technique and validates the technique being presented with figures and tables.

We have updated the manuscript as indicated beginning at line 312.

11. Please include a disclosure section as well. If no disclosures then please write none.

We have added a disclosure section; see line 437.

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.

We have updated the video as suggested.

2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol.

We have updated the narration to match the written protocol.

3. Please do not use personal pronouns in the narration. All narration should be in imperative tense as if describing someone how to perform the technique.

We have updated the usage in the narration to reflect this requirement.

4. Please ensure that the protocol subsection titles are the same both in the text and the video.

We have updated the subsection titles to more accurately reflect the written protocol. An example of this is the “Hydrogel and Lipid Deposition” subsection title card.

5. Title Cards:

• Please add a Main title card at the beginning, that lists the title of the protocol, the authors, and their affiliations. Check for the Jove standard guideline for title cards
• Please capitalize the first letter of every important word in your title.
• Please use Super script for the Affiliation number.
*• Please Use an asterisk * for noting authors contributing equally.*
• Please remove the title of the protocol from every chapter cards.
• Please capitalize the first letter of every important word in your Chapter title cards.

We have updated the main title card as indicated and adjusted the text in the chapter title cards to reflect the last two points.

6. Video Editing Content:

• Consider making the protocol section into parts and adding specific protocol methods with chapter cards. it will be easier to understand.

We have adjusted the organization of the video as recommended. An example of this is indicating the Hydrogel and Lipid Deposition section

7. Audio Editing and Pacing:

• Audio are quite low and not balanced. Please ensure audio level peaks average around -9 dB.

We have balanced the audio levels as suggested.

Review Response to Construction of Model Lipid Membranes Incorporating G-protein Coupled Receptors (GPCRs)

Ahmed Elbaradei, Lucia C. Dalle Ore, Noah Malmstadt

Email: malmstad@usc.edu

Response to Reviewers:

We appreciate the opportunity to revise our work for submission to the Journal of Visualized Experiments. We are grateful for the time and consideration the reviewers have given to providing valuable feedback on our manuscript and video. In this response, reviewers' comments are in *blue text* and our response are in black text.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The work "Hydrogel swelling to incorporate G protein-coupled receptors in giant unilamellar vesicles" by Elbaradei et al. describes in detail a protocol for generating GUVs having transmembrane proteins embedded on the GUV membranes. This works is especially interesting for the research field given that although GUVs have been intensively used as a model membrane system to investigate proteins' functions in physiologically relevant membrane environments, methods to robustly generate GUVs incorporating transmembrane proteins are missing. By modifying the hydrogel swelling method developed by Horger et al., the authors robustly generate GUVs with transmembrane proteins.

We are grateful for the time and consideration the reviewer has given our manuscript.

Major Concerns:

1. How do the authors know the orientation of the proteins (for instance as drawn in Fig. 1)? Given the method described here, one would expect the orientation of the proteins are half facing inward and half facing outward. It would be important that the authors explain the experiments/results showing the proteins are oriented correctly.

We have shown this to be the case in previous work; we dye-labeled an antibody to a cytosolic loop of the adenosine receptor ($A_{2A}R$) and incubated the labeled antibody with the protein, then incorporated the labeled protein into lipid-dye-labeled GUVs using the modified hydrogel swelling method.[1] We then exposed the protein-incorporated vesicles to a charged quencher, which is unable to cross the bilayer. This results in a 50% reduction in fluorescence of the lipid dye, but the fluorescence of the labeled protein remains unaffected by the quencher, demonstrating orientation as in a cell. We have assessed 5-$HT_{1A}R$ orientation using the same method.[2] We have added these details the discussion; see line 370. The relevant citations are references 44 and 45.

2. How do the authors assess protein functionality, especially the gel-protein mixtures are heated to 45C, which could cause protein degradation. It would be informative that the authors describe briefly about the methods they used to assess protein functionality. Also, would this hydrogel method works if one prepare and use the hydrogel-protein mixture at a lower temperature, for instance at room temperature or 4°C?

Protein functionality has been assessed in previous by encapsulating BODIPY-GTPγS, which binds with the $G_{i\alpha}$ subunit in the active state of the receptor, into GUVs. Binding unquenches the fluorophore producing a fluorescence signal.[2] We also have included a description of this experiment in the introduction: line 163, reference 45.

We use an ultra-low melting temperature agarose that has a gelling temperature of 8-17 °C and a melting temperature of 50 °C. We need to keep the agarose at higher temperature than its gelling temperature to maintain a homogenous mixture of the protein and the agarose and to easily produce the agarose film. We have included a discussion of this in the manuscript starting at line 401.

3. A major concern of using agarose is that the gel is trapped inside the resulting GUVs or embedded on GUV membranes, as shown in [1]. The presence of the agarose gels on GUV membranes can thus alter the mechanical properties of the membrane [1], which in turn the functionality of the proteins. The authors should comment on this issue.

[1] Giant unilamellar vesicles formed by hybrid films of agarose and lipids display altered mechanical properties. R. Lira, R. Dimova, and K. Riske. DOI: 10.1016/j.bpj.2014.08.009

We use the modified hydrogel swelling method described herein to prepare GUVs for micropipette aspiration (MPA) experiments. A phase contrast image of a hydrogel swelling-produced vesicle being deformed by suction into a glass capillary is shown below. A vesicle that is filled with agarose would not be able to deform in this manner. We have included this in the discussion starting at line 415.

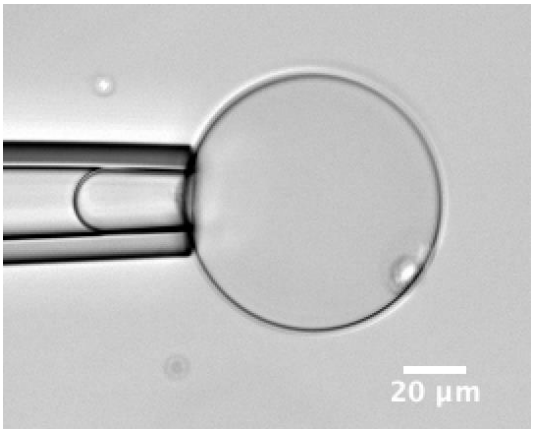

Figure 1. Phase contrast image of a GUV fabricated from 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) using the agarose swelling method described herein being aspirated by a glass capillary.

Minor Concerns:

Line 259: for readers not in the specific research field of 5-HT1A, it would be helpful to describe very briefly what is "5-HT1A membrane prep", especially this protein is purified from mammalian cell membranes but not from bacteria (which would be covered by detergent after purification or proteoliposomes, that are commonly used for purifying transmembrane proteins). Does the method describe here can only be used for proteins purified from cell membranes? Would the method work if uses proteoliposomes?

We apologize for the confusion, “Membrane prep” is a suspension of fragmented membranes containing the protein of interest. Membrane fragments are prepared from mammalian cell membrane of a HEK293 cell line which has been modified to overexpress the receptor and cognizant G proteins. We have changed “membrane prep” to “membrane fragments” for clarity; see line 192.

While we have not worked with proteoliposomes, we have worked with both purified proteins from mammalian cell membranes and membrane fragments.[1] For purified detergent solubilized proteins, diluting the protein solution during the swelling step was sufficient to transfer the protein from the detergent into the membrane of GUVs.[1] (Reference number in the main text is 45).

Line 139: I suggest to change "in isolation" to "in membrane environment"

We have changed “in isolation” to “the membrane environment” for clarity; see line 149.

line 145: it is unclear what "intrinsic activity" meant here. Does it refer to protein functionality? It would be helpful for the readers if the authors could clarify this point.

We meant to describe the ability of this technique to assess the basal activity of the protein or the ability of the receptor to activate intracellular signaling pathways in the absence of ligand. However, we agree with the reviewer that “intrinsic activity” in this context would be confusing and we have changed “intrinsic activity” with “protein functionality”; see line 158.

Line 281: given that methanol is toxic and volatile, the authors should mention that one should handle methanol in a suitable chemical hood

We have added a sentence indicating this in the note of step 2.1, line 217.

Line 300: how long can one keep the agarose solution at 45°C? In other words, how often should one make new gel solution?

The protein-free agarose solutions can be kept on a heating block for at least 48 hours. We have added a sentence indicating this in the note of step 3.1, line 235.

Line 304: why use different percentages of agarose for making GUVs with and without proteins? It would be informative to give the rationale behind the choice.

The addition of protein solution to the agarose solution brings the agarose concentration to that of the protein-free solution. Upon gel swelling, both solutions have the same agarose concentration. We have added a sentence clarifying this in line 392.

Line 321: given that chloroform is toxic and volatile, the authors should mention that one should handle the solvent in a suitable chemical hood

We have added a sentence indicating this in the note of step 6.1, line 263.

Reviewer #2:

Manuscript Summary:

Transmembrane proteins such as GPCRs play a key role in cell-to-cell communications and they are involved in various cellular functions. However, investigating their function-structure relationships remains challenging as it requires a lipid bilayer membrane for their activity and stability. As a result, studying membrane proteins in vitro using conventional approaches is formidable. To overcome these bottlenecks, various synthetic models which mimics the plasma

membrane such as liposomes (SUV, LUVs, and GUVs) and nanodiscs were employed to study protein-protein and protein-lipid interactions (Sarkis et al., 2020). Therefore, incorporating proteins to GUVs using agarose swelling approach is of significance.

In this Manuscript and video, Elbaradei et al. report a generalized method that can be used to reconstitute proteins in GUVs. The authors provide a clear step-by-step protocol that can be applied for reconstitution of other proteins. It is apparent that this work, regardless of its limitations, represents a broad impact and significance to study other proteins and investigate how lipids regulate their activity.

We appreciate the positive feedback and helpful suggestions from the reviewer. We are grateful for the time and comments the reviewer has given our manuscript.

Major Concerns:

1. The prior literature should be included to help readers understand and apply this method for protein-lipid interaction studies.

Lipid-protein interactions can be investigated by a number of different techniques such as surface plasmon resonance (SPR),[3, 4] nuclear magnetic resonance spectroscopy (NMR), [5, 6]protein lipid overlay (PLO) assay,[6–9] native mass spectrometry,[10] isothermal titration calorimetry (ITC),[11, 12] and liposome sedimentation assay.[13, 14] Also, various studies investigated the role of proteins in sensing or stabilizing membrane curvature.[15, 16] Our lab developed the GUV approach to study the effect of lipid-protein interactions on protein functionality by incapsulating BODIPY-GTPγS which binds with the $G_{i\alpha}$ subunit in the active state of the receptor. Their binding unquenches the fluorophore producing a fluorescence signal that could be detected over time.[2] We have also added context in the introduction starting on line 159.

2. Once the protein is reconstituted, the storage conditions and its stability should be stated.

The protein-incorporated GUVs are always observed on the same day in which they are prepared. We have added a sentence indicating this in the protocol section, step 12, line 307.

3. The authors should describe briefly why they choose 5-HT1AR protein for reconstitution.

We used human serotonin 1A receptor (5-HT_{1A}R) because it is a prototypical G protein-coupled receptor. 5-HT_{1A}R influences numerous physiological functions such as cardiovascular, neurological, gastrointestinal, and endocrine functions.[17] We have added context indicating this in the introduction; see line 153 and forward.

4. In fig3, it is not clear how the line intensity dataset is collected and needs to be described if a DLS or other instruments are used.

The images in Figure 3 a, and b were taken using a spinning disk confocal microscope. Line intensity profiles were plotted below the microscope images. The line intensity profile shows a two-dimensional plot of the intensities of the pixels along the white dashed line within the image. The x-axis would be the distance along the line and the y-axis is the pixel intensity. Using this intensity profile we could assess the fluorescence signal resultant from the rhodamine-labeled protein and evaluate its incorporation in the membrane. ImageJ software was used to plot the profile intensity of the indicated line. We have also added a sentence indicating this in the representative results section; see line 321.

Minor Concerns:

1. Describe the labeling efficiency based on the current or prior studies.

The labeling efficiency was calculated as the molar ratio between the dye and the protein to be 1.1. By measuring the absorbance of the protein at 280 nm and the Rhodamine absorbance at 554 nm, we can calculate the concentration of the protein and the degree of labeling as follows:

Protein concentration (M) = $[A_{280}- (A_{max} \text{ X } CF))/(\varepsilon_{protein})] \text{ X Dilution factor}$

Moles of dye per more protein = $[A_{max}/ (\varepsilon' \text{ X protein concentration (M)}] \text{ X Dilution factor}$

Where, ε protein = protein molar extinction coefficient, $A_{max} = A_{554}$

CF = Correction factor $=A_{280}/A_{max}$; CF for Rhodamine = 0.3400

ε´ = Rhodamine molar extinction coefficient = 80,000 M-1 cm-1. The calculations provided are courtesy of the manufacturer, Thermofisher, instruction manual. We have added context indicating this in the representative results, see line 312

2. In line 346, though BSA is used to prevent unspecific binding, it is worth mentioning it in a note.

We have added a sentence indicating that in the note of step 9.1, line 284.

3. In line 354, indicate from which step the procedure is continued.

We have added an indication at step 10, 288.

References:

1. Gutierrez, M.G. *et al.* The lipid phase preference of the adenosine A2A receptor depends on its ligand binding state. *Chemical Communications*. **55** (40), 5724–5727, doi: 10.1039/C8CC10130B (2019).
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5. Brown, M.F., Miljanich, G.P., Franklin, L.K., Dratz, E.A. H-NMR studies of protein-lipid interactions in retinal rod outer segment disc membranes. *FEBS letters*. **70** (1), 56–60, doi: 10.1016/0014-5793(76)80725-9 (1976).
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