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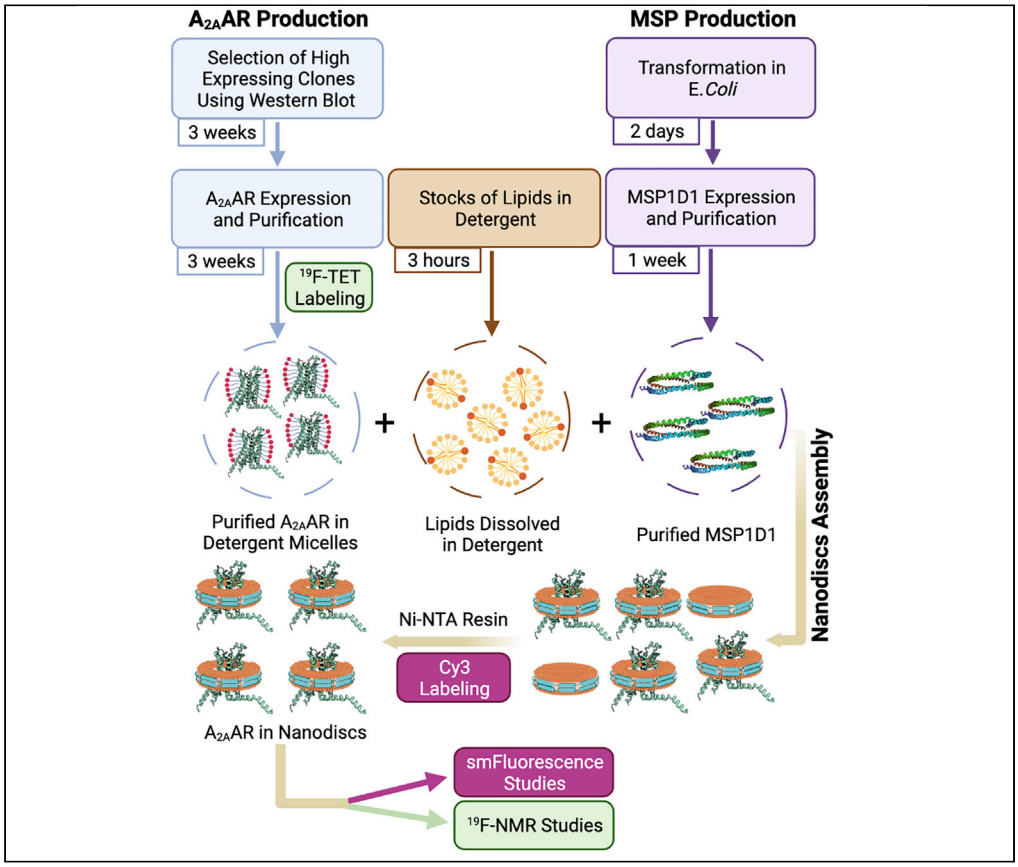
Production of human $A_{2A}AR$ in lipid nanodiscs for ^{19}F-NMR and single-molecule fluorescence spectroscopy



We describe production of the human A_{2A} adenosine receptor ($A_{2A}AR$), a class A G protein-coupled receptor (GPCR) for ^{19}F-NMR and single-molecule fluorescence (SMF) spectroscopy. We explain in detail steps shared between the two sample preparation strategies, including expression and isolation of $A_{2A}AR$ and assembly of $A_{2A}AR$ in lipid nanodiscs, and procedures for incorporation of either ^{19}F-NMR or fluorescence probes. Protocols for SMF experiments include sample setup, data acquisition, data processing, and error analysis.

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Highlights

Detailed instructions for human $A_{2A}AR$ expression in *P. pastoris*

Step-by-step protocol for assembly of lipid nanodiscs containing $A_{2A}AR$

Incorporation of chemical probes for NMR and single-molecule fluorescence

Single-molecule fluorescence sample assembly and data acquisition

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Production of human $A_{2A}AR$ in lipid nanodiscs for ^{19}F-NMR and single-molecule fluorescence spectroscopy

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SUMMARY

We describe production of the human A_{2A} adenosine receptor ($A_{2A}AR$), a class A G protein-coupled receptor (GPCR) for ^{19}F-NMR and single-molecule fluorescence (SMF) spectroscopy. We explain in detail steps shared between the two sample preparation strategies, including expression and isolation of $A_{2A}AR$ and assembly of $A_{2A}AR$ in lipid nanodiscs and procedures for incorporation of either ^{19}F-NMR or fluorescence probes. Protocols for SMF experiments include sample setup, data acquisition, data processing, and error analysis.

For complete details on the use and execution of this protocol, please refer to Wei et al. (2022) and Sušac et al. (2018).

BEFORE YOU BEGIN

The following protocol details steps for preparing samples of human $A_{2A}AR$ in lipid nanodiscs for ^{19}F-nuclear magnetic resonance (NMR) and total internal reflection single-molecule fluorescence (SMF) spectroscopy, which share some sample preparation steps. We also report details on SMF data acquisition and analysis. First, we describe steps for expressing an $A_{2A}AR$ variant in *Pichia pastoris* containing a single extrinsic cysteine enabling incorporation of either a fluorescent label or ^{19}F-NMR probe at a judiciously selected location within the receptor (Sušac et al., 2018). Protein production starts with selection of a high-expressing clone, followed by large-scale expression and purification in aqueous solutions containing mixed micelle detergents. We then describe protocols to assemble samples of lipid nanodiscs containing purified $A_{2A}AR$, including preparation of the membrane scaffold protein MSP1D1 (Denisov et al., 2004). Incorporation of ^{19}F-2,2,2-Trifluoroethanethiol (TET) for NMR experiments, utilizing in-membrane chemical modification (Sušac et al., 2015, 2018), or a Cyanine 3 maleimide (Cy3) dye for SMF experiments is performed at different steps and described in detail. Finally, we explain steps to mount $A_{2A}AR$ samples for SMF experiments, acquire TIRF data and quantitatively analyze fluorescence intensities. The described protocols complement previously reported sample preparation methods for SMF experiments (Lamichhane et al., 2017). The current protocol uses shake flask production with *P. pastoris*, which we found provides several important advantages. Alternative approaches include production in a bioreactor (Singh et al., 2008) or using *Saccharomyces cerevisiae* (Wedekind et al., 2008). To get started, prepare the required buffers and cell culture media as described in the "materials and equipment" section.

Identification of colonies with high expression levels of the A_{2A} adenosine receptor ($A_{2A}AR$) in *Pichia pastoris*

Timing: 13–15 days

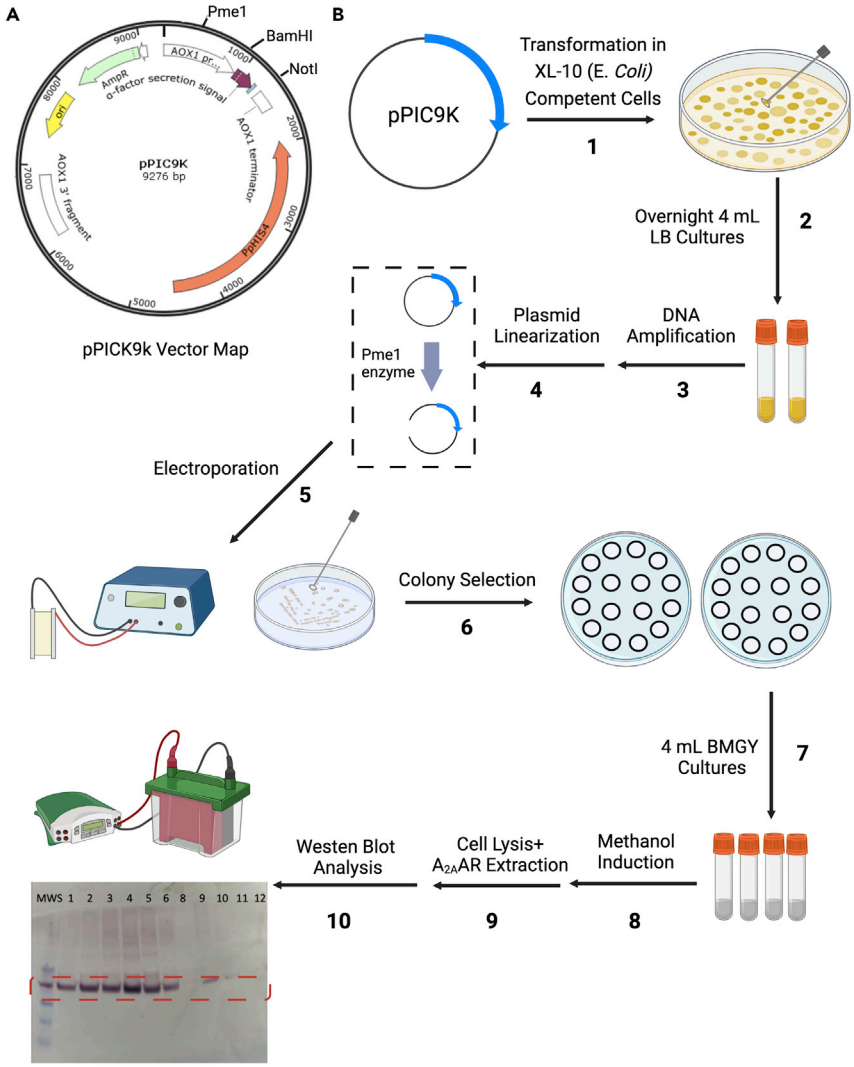

Figure 1. Identification of colonies with high expression levels of $A_{2A}AR$ in *Pichia pastoris*

(A) pPIC9K vector diagram. Relevant regions are annotated, including the AOX-1 promoter, AOX-1 terminator, origin of replication, antibiotic resistance, and the PME1, BamHI and NotI restriction sites.

(B) Optimized workflow to identify *Pichia pastoris* clones with high levels of $A_{2A}AR$ expression. Individual steps are numbered and described in further detail in the text.

Figure created with BioRender.com.

Refer to Figure 1 for Steps 1 to 24

1. Verify plasmids: Obtain the plasmid (Figure 1A) from the corresponding authors (Wei et al., 2022) or clone the ADORAA2A gene. The gene contains a variant of human $A_{2A}AR$ (1–316) with a point mutation to remove the only glycosylation site (N154Q), an N-terminal FLAG tag, a C-terminal 10× HisTag (deca-HisTag), and the amino acid mutation A289C. This gene was inserted into a pPIC9K vector (Invitrogen) at the BamHI and NotI restriction sites.

   Note: This is a low copy plasmid and sufficient amounts of the plasmid (> 20 μg) should be generated using a HiSpeed plasmid midi-prep kit (Qiagen) or equivalent method.

2. Generate and validate plasmid (Figure 1B): Follow the manufacturer's protocol for generation of purified plasmid from *E. coli* such as TOP10 competent cells (ThermoFisher) cultured in LB media.

https://www.thermofisher.com/us/en/home/references/protocols/cloning/competent-cells-protocol/routine-cloning-using-top10-competent-cells.html Use the AOX1 promoter and AOX1 terminator as forward and reverse primers, respectively, to sequence the plasmid with an appropriate sequencing service. Store the purified plasmid at −20°C.

Note: We adopted one alternative step from the Qiagen midi-prep protocol that we found to be helpful. Specifically, elute the purified plasmid in ~700 μL of doubly distilled H_2O (ddH_2O). This smaller elution volume can prevent the need to further concentrate the plasmid in downstream steps and results in a typical concentration of ~200 μg/mL plasmid.

3. Plasmid linearization: Mix 20 μg of plasmid (e.g., 100 μL at 200 μg/mL) with 20 μL of Pme1 enzyme (10 U/μL) (NEB) and 10 μL of CutSmart Buffer (NEB). Incubate the sample for 1 h at 37°C and 700 rpm shaking.

   Note: If the plasmid concentration is higher, add sterile ddH_2O to bring the final solution volume up to 130 μL.

4. Plasmid purification: Precipitate the linearized DNA by adding ice cold 3 M sodium acetate pH 5.2 at 1/10 of the volume of sample from step 3 and mix well using a vortexer for 30 s. Then add ice cold 100% ethanol at 3 times the volume of this sample. Using the volumes provided in step 3, this corresponds to the addition of 13 μL sodium acetate and 429 μL ethanol. Mix well again using a vortexer and incubate the sample on ice for 10 min.

   Note: After the addition of 100% cold ethanol, a white precipitate should be visible in the solution.

5. Wash the purified plasmid: Pellet the sample at 18,000 × *g* at 4°C for 10 min. Discard supernatant and add 1 mL of 70% ethanol. Resuspend the plasmid by mixing well with a vortexer. Spin down the sample again at 18,000 × *g* at 4°C for 10 min. Discard supernatant.

   Note: The plasmid should now appear as a small white pellet at the bottom of the tube.

6. Dry and dissolve the plasmid: Gently remove any remaining ethanol using a bench vacuum line attached to a 1 mL micropipette tip. Be careful not to perturb the pellet or excessively dry out the pellet. Dissolve the pellet in 70 μL of sterile ddH_2O water.

   Note: Check the plasmid concentration by measuring the absorbance at 260 nm. The plasmid concentration should be between 200 to 300 ng/μL. If needed, increase the concentration of the plasmid using a centrifugal evaporator or equivalent method.

7. Preparing competent cells for electroporation: Add 5 μg of linearized plasmid to 60 μL of electrocompetent *P. pastoris* cells in a 0.2 cm electroporation cuvette (BioRad). Mix by gently pipetting (up and down). Incubate on ice for 10 min.

   Note: For $A_{2A}AR$ expression, we typically use BG12 electrocompetent cells provided by BioGrammatics.

8. Electroporation and recovery: Wipe off any condensation from the cuvette with a towelette. Place the cuvette into the electroporator and electroporate the cuvette using the "Fungi-Pic" program on a Bio-Rad MicroPulser electroporator or using an equivalent instrument. As with all other steps in this protocol, use basic sterile laboratory techniques. Immediately after electroporation, add 1 mL of cold 1 M sorbitol, mix by gentle pipetting and transfer the solution to a culture tube. Incubate the solution at 30°C for 1 h with 200 rpm shaking.

Note: A single pulse of 2 kV voltage was applied for the "Fungi-Pic" program.

9. Plating the solution containing transformed cells: Pipette 50–100 μL of the solution containing the transformed cells onto minimal media plates containing carbenicillin and spread evenly using sterile glass beads.

Note: To prepare the minimal media plates, the steps are:

a. Dissolve 2 g of dextrose with 1.5 g of agar in 90 mL of water then autoclave.
b. Dissolve 170 mg of YNB with 50 mg of ammonium sulfate in 9.8 mL of water; add 200 μL of 0.02 g / 100 mL biotin. The medium can be stored at 4°C.
c. Let the autoclaved solution cool on a lab bench. The media should be warm enough to still be a solution but cool enough that one could place their hand on the media bottle without burning it. To qualitatively assess the temperature, lightly place a finger or two on the media bottle exterior, taking care not to burn your fingers.
d. Once the media has cooled and before it has solidified, add the 10 mL YNB solution from step 9(c) and 100 μg/mL carbenicillin.
e. Prepare plates by pipetting into sterilized petri dishes, cover and allow to solidify. Plates will be ready to use in about one hour after distributing the media.

Note: The optimal volume of solution can sometimes vary from one electroporation event to another. We typically pipette two different volumes onto two different plates: one volume of ~20 μL and a second of 75–100 μL. The transformed plates should contain numerous colonies that are isolated but should not be crowded. Only consider selecting colonies that are not overlapping or very near others.

Note: The pPIC9k vector is His4- and the BG12 cells are His4+. Thus, minimal media plates can be used for selection of cells containing the correct plasmid, as non-transformed cells will not grow on the minimal media plates.

Note: The protocol for preparing the minimal media plates can be found in the material and methods section.

10. Maturation of plates containing transformed cells: Incubate the plates at 30°C until colonies are visible. Colonies typically appear within 36 h and the plates are ready to be removed from the incubator after 48–72 h.

Note: After removing plates from the incubator, they can be stored in at 4°C for up to 1–2 months to maintain viability of most colonies.

11. Colony selection and plating: Select 20–25 isolated colonies from the plate containing transformed cells. Using a sterile inoculating loop, transfer each colony to a distinct individual area of a new minimal media plate. With the same inoculating loop, spread out the colony in a circle with a radius of about 1 cm, accounting 8–10 unique areas per plate. Use a new inoculating loop for each colony. Incubate the plates at 30°C until the areas to which colonies were spread are dense with cell growth (2–3 days).

Note: From our experience, a higher-expressing clone is typically identified among 20 to 25 selected colonies. If a higher expressing colony is not identified among that many, one can screen additional colonies, if desired. However, from experience this does not typically result in identification of a colony expressing the protein of interest.

Note: Like the first plates used in this protocol, these plates can be stored at 4°C for up to 1–2 months to maintain viability of most colonies.

12. Culturing colonies to test for level of protein expression: Using a sterile inoculating loop, scrape cells from an individually numbered area on the minimal media plate and inoculate 4 mL of buffered minimal glycerol (BMGY) media in a standard culture tube. Repeat this for all numbered colonies. Place the tubes in an incubator shaker and allow the cultures to grow for 2 days at 30°C and 200 rpm shaking.

Note: See Materials and methods for the protocol to prepare BMGY media.

Note: Before proceeding to the next step, make sure the cultures appear dense and milky white in color. The cultures should reach an OD_{600} of at least 12–15 before proceeding with the next step.

13. Prepare the cells for protein expression: Spin down the cells at 3,000 × *g* and 4°C for 15 min and discard the supernatant. Resuspend the cells in 4 mL of **BMMY** (Buffered minimal methanol) media (see materials and equipment) without methanol by vortexing. Incubate the cultures for 6 h at 28°C and 200 rpm shaking.

Note: The initial exclusion of methanol in the media allows the cells to digest any remaining glycerol, which can inhibit protein expression.

14. Induce protein expression: Induce protein expression by adding 200 µL of 100 mg/mL methanol (5 g/L final concentration) to the 4 mL cultures. Add the same volume and concentration of methanol two more times, ~12 and ~24 h after the first methanol addition. After the final methanol addition, allow the cultures to grow for an additional 16 h at 28°C.
15. Harvest the cells: Pellet the cells in a tabletop or floor centrifuge at 3,000 × *g*, 4°C for 15 min. Discard supernatant and store the cells at −80°C or proceed immediately to the next step.

Pause point: Cell pellets can be kept frozen for extended periods of time. However, the corresponding colonies from which the cells were initially taken have a shelf life of 1–2 months. We therefore do not recommend waiting long than this time to proceed to the next step.

16. Prepare the cells for small-scale lysis: If frozen, thaw the cells on ice. Resuspend the cells with 200 µL of **A_{2A}AR High Salt Buffer** by pipetting or vortexing.
17. Lyse the cells: Fill a 2 mL conical tube with 1 mL of glass beads. Add the resuspended cells to the tube. Lyse the cells using a bead beater such as a Digital Disruptor Shaker (Scientific Industries) set to 3000 rpm for 10 min or use an equivalent instrument that provides rapid shaking such as a bead mill homogenizer.

Note: A bead beater is required for applying the necessary force to break open the yeast cell outer walls. A hand homogenizer cannot be used here effectively.

18. Extract the receptor: Add 500 µL of the cell lysate and 500 µL of solubilization buffer to a 1.7 mL conical tube. Incubate the samples for 2.5 h at 4°C with rotation.
19. SDS-PAGE sample preparation: Centrifuge the tubes containing the solution from step 18 at 15,000 × *g* for 30 min at 4°C and collect the supernatant. Collect 25 µL from each tube to run as a sample on an SDS-PAGE gel. Add 25 µL of **Gel Loading Buffer (2×)** to each sample and vortex. Run the samples on an SDS-PAGE gel. We typically apply 120 V for 45 min.

Note: Do not heat the samples for SDS-PAGE analysis. From our experience, heating GPCR samples for SDS-PAGE produces many aggregates of different multiples of the protein molecular weight, which are not actually present in the sample.

20. Set up the western blot: Prepare the "**Blocking Solution**". Transfer the contents of the SDS-PAGE gel to the western blot paper using an iBlot2 instrument (ThermoFisher) at 20 V for 5–7 min or use an equivalent device to transfer the proteins to western blot paper. Treat the western blot membrane for 1 h at 20°C–23°C with the blocking solution, then decant the solution.
21. Add the antibody: Wash the western blot membrane for 10 min at 20°C–23°C with the **"Washing Solution".** Decant the solution. Add the **"Antibody solution"** and incubate the western blot membrane for 1 h at 20°C–23°C or 12–14 h at 4°C). Then remove the solution and store it into a small container at 4°C.
22. Remove non-specific interactions: Wash the western blot membrane for 10 min at 20°C–23°C with 20 mL of the **"Washing Solution".** Decant the solution and repeat this process two more times.
23. Develop the western blot membrane: Prepare the **"Developing Solution"** by crushing one tablet (Sigma Fast BCIP/NBT) and dissolving it in 15 mL of water. Develop the western blot by adding the developing solution and gently rocking it. After bands appear, discard the developing solution, and rinse the membrane with water several times.

Note: It takes 2–3 min for bands to appear. This time may vary depending on the level of expression.

24. Analyze the western-blot results and select the high-expressing clones based on the intensities of observed bands. Prepare the glycerol stocks of these high-expressing clones and store them at – 80°C.

Large scale expression of A_{2A}AR in P. *pastoris*

Timing: 2 weeks

Refer to Figure 2 for the following Steps 25 to 31.

25. Start initial cultures: Inoculate 4 mL buffered minimal glycerol (BMGY) media in 15 mL culture tubes with 300 µL solution of resuspended glycerol stocks. Let the culture incubate at 30°C for 48 h to reach an optical density of 9–10.
26. Scale up the cultures to 50 mL: Inoculate 50 mL BMGY medium in a 250 mL baffled flask with the 4 mL cultures. Incubate at 30°C with shaking at 200 rpm for approximately 60 h to reach an optical density of 15–20.
27. Scale up the cultures to 500 mL: Inoculate 500 mL BMGY media in a sterile 2.8 L baffled flask with one 50 mL culture. Incubate at 30°C for 48 h to reach an optical density of 15–20.
28. Transfer the cells into BMMY media: Spin down the cell cultures at 3,000 × *g* for 15 min. Discard the supernatant and resuspend the cells in 500 mL of buffered minimal methanol (**BMMY**) medium without methanol by vortexing. Add the resuspended cell culture into a new sterile 2.8 L baffled flask. Lower the temperature of the incubator shaker to 28°C.
29. Digest remaining glycerol: Incubate the cultures at 28°C with shaking for 6 h to ensure complete metabolic digestion of glycerol before methanol addition.
30. Induce protein expression: Prepare a solution containing 10% methanol in ddH_2O (w:v) and sterile filter. Add an aliquot of the solution to the flask containing the cell culture to reach a final concentration of 5 grams methanol for every 1 L cell culture. Add the methanol solution at two additional times with 12-h intervals after initial induction. After the final addition of methanol, let the cultures grow for 12 h at 28°C.
31. Harvest cell culture: Centrifuge the cell culture at 3,000 × *g* for 15 min. Discard the supernatant. Freeze the cell pellets in liquid nitrogen and store them at −80°C for later use.

Note: The expected yield is about 35–40 grams of wet cell pellet per liter of culture.

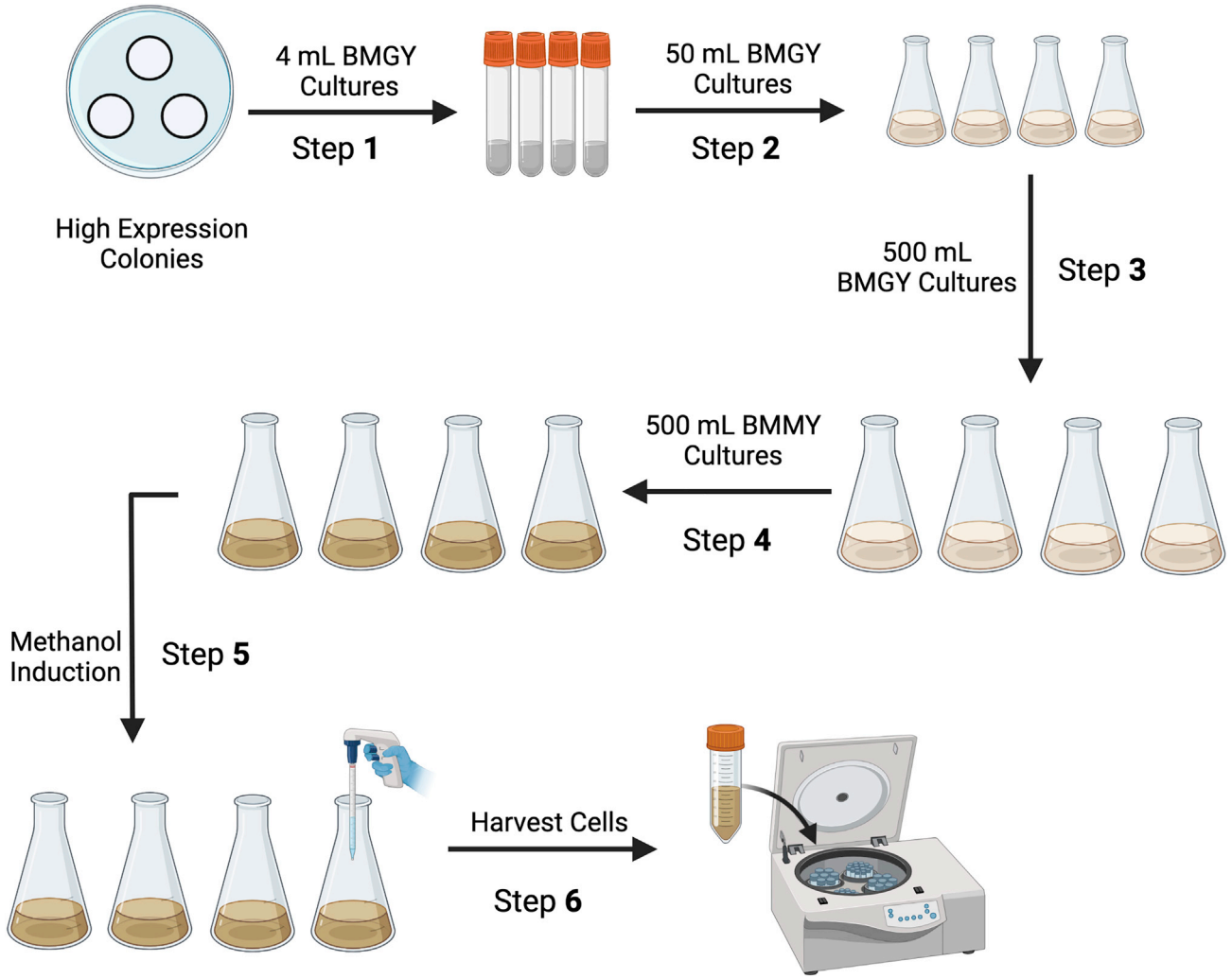

Figure 2. Workflow for large-scale expression of $A_{2A}AR$ in *Pichia pastoris*

The workflow above starts with colonies identified from the process described in Figure 1. Individual steps are numbered and described in further detail in the text. Figure created with BioRender.com.

Purification of $A_{2A}AR$ for nanodisc assembly

Timing: 2 days

Refer to Figure 3 for Steps 32 to 41

32. Lyse the cells: Resuspend the cell pellets containing $A_{2A}AR$ in **$A_{2A}AR$ Lysis Buffer** and in-house prepared protease inhibitor solution and lyse by a single pass through a cell disruptor (Pressure Biosciences) operating at 40,000 PSI.

Note: To check that the cells were lysed, allow the product collected from the cell disruptor to sit untouched on ice or in a cold room for about 15 min. The solution should appear to separate into two different components, one appearing more cloudy than the other.

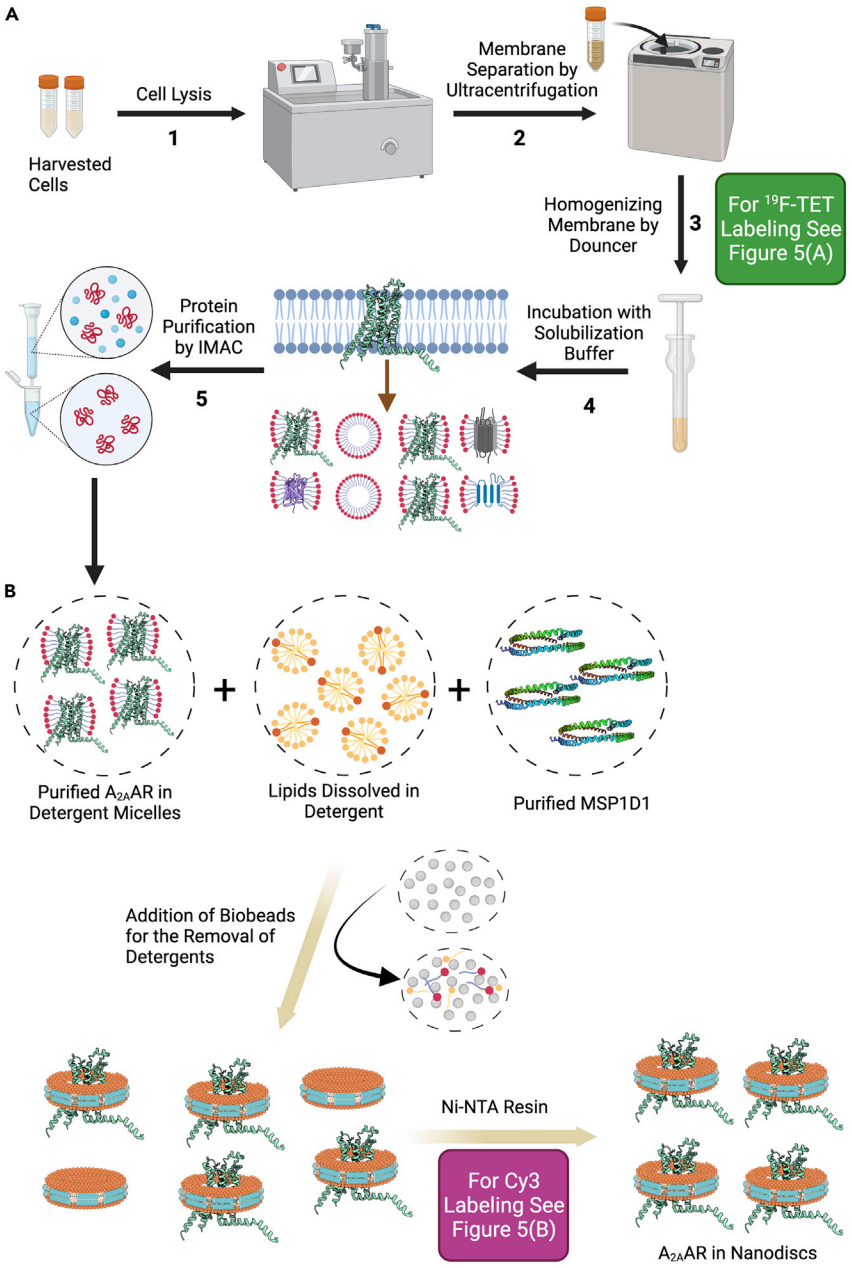

Figure 3. Workflow for extraction and purification of A_{2A}AR with detergent micelles and reconstitution into lipid nanodiscs

(A) The optimized workflow for A_{2A}AR extraction with mixed detergent micelles and purification starts with cell cultures containing A_{2A}AR, which were obtained at the end of the process shown in Figure 2. Individual steps are numbered and described in further detail in the text. The green box indicates the point in this process where ^{19}F-TET labeling would be done to produce NMR samples. This step is expanded in Figure 5A.

(B) Stepwise procedure for the formation of nanodiscs containing A_{2A}AR. Individual components are mixed, and the formation of nanodiscs is initiated by the addition of detergent-removal Biobeads. The purple box indicates the step at which a Cy3 cysteine-reactive fluorophore is added for single-molecule fluorescence experiments. This step is expanded in Figure 5B.

Figure created with BioRender.com.

33. Isolate the membranes: Spin down the lysed sample by ultracentrifugation at 200,000 × *g* at 4°C for 30 min and decant the solution. Freeze the membranes in liquid nitrogen and store at −80°C for future use or proceed immediately to the next step.

Note: For ^{19}F-TET labeling, please refer to section **A_{2A}AR ^{19}F-Trifluoroethanethiol (TET) labeling for NMR experiments** in step-by-step method details.

34. Resuspend the membranes: If working with frozen membrane pellets, allow them to gently thaw. Resuspend the isolated membrane pellets in **A_{2A}AR High Salt Buffer** and add 1 mM theophylline and protease inhibitor using 100 mL glass tissue homogenizer (Sigma). Allow the resuspended membranes to incubate with the buffer on the stirrer for 1 h at 4°C.
35. Wash the membranes: Spin down the membrane at 200,000 × *g* for 30 min at 4°C. Remove the supernatant and homogenize the membranes again in **A_{2A}AR High Salt Buffer** using the glass tissue homogenizer.
36. Solubilizing the membrane: Add **A_{2A}AR Solubilization Buffer** to the homogenized membranes. Let the sample incubate for 6 h at 4°C with gentle mixing.

Note: For small volumes (e.g., 100 mL or less), the membranes and buffer mixture can be placed in 50 mL conical tubes and allowed to rotate gently at 4°C. For larger volumes (>100 mL), the mixture can be placed in glass bottles with a stir for gentle mixing.

37. Collect the solubilized receptor: Prepare Co^{2+}-charged affinity resin (TALON, Takara Bio USA) by washing with 5 column volume (CVs) of ddH2O, then 5 CVs of **A_{2A}AR High Salt Buffer.** Separate the insolubilized material by ultracentrifugation at 200,000 × *g* for 30 min and collect the supernatant. Add the supernatant to the Co^{2+}-charged affinity resin in a conical tube and add imidazole to 30 mM final concentration to prevent non-specific binding. Incubate 12–14 h at 4°C with gentle stirring.

Note: 2 mL of Co^{2+}-charged affinity resin is used for 1 L of culture.

Note: To prepare a concentrated stock solution of imidazole, take care to carefully adjust the pH to 7.5 using NaOH or other appropriate base. As the imidazole solution is sensitive to light, be sure to store it in the dark or dimly lit location if kept for more than a few days.

38. Remove impurities: Spin down the resin at 1,500 × *g* for 10 min. Decant the solution and add 20 CVs of **A_{2A}AR Wash Buffer 1.** Gently resuspend the resin by inverting the conical tube and allow to rotate at 4°C for 1 h. Repeat this process using 20 CVs of **A_{2A}AR Wash Buffer 2** followed by 20 CVs of **A_{2A}AR Wash Buffer 3.**
39. Elute the protein: Elute pure A_{2A}AR with 5 CVs of **A_{2A}AR Elution Buffer.** Perform the elution in steps of 0.5 CV. Keep the fractions.

Note: Typically, most of the receptor will be contained in fractions 2–4 if using 0.5 CV elution steps.

40. Exchange the buffer: After elution, exchange the protein into **A_{2A}AR Desalting Buffer** using a PD-10 desalting column (Cytiva) following the manufacturer's protocol. https://cytiva-delivery.sitecorecontenthub.cloud/api/public/content/digi-11531-pdf.
41. Concentrate the receptor: Concentrate purified A_{2A}AR at 4°C to the desired concentration required for the nanodiscs preparation using a Vivaspin-6 centrifugal concentrator with a 30 kDa MWCO.

Note: For concentrating A_{2A}AR, we recommend not exceeding 300–400 × *g* centrifugal force, as higher applied force can result in protein aggregation. We recommend using intervals of 20–25 min to concentrate the protein, with gentle but thorough mixing of the sample in between each interval.

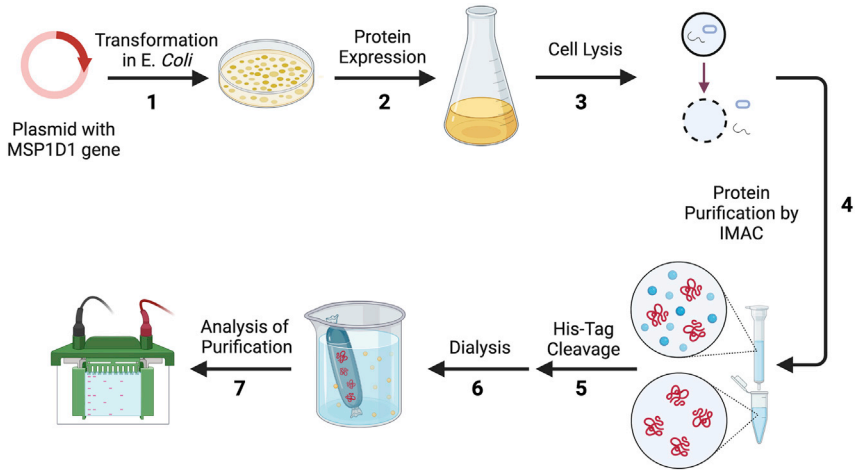

Figure 4. Workflow for the expression and purification of the membrane scaffold protein MSP1D1
Individual steps are numbered and annotated. The figure is adapted from "Protein Overexpression and Purification from Bacteria" by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

Note: $A_{2A}AR$ should be purified fresh for every nanodiscs assembly preparation.

Expression and purification of the membrane scaffold protein MSP1D1 for nanodisc production

Timing: 5 days

Refer to Figure 4 for Steps 42 to 53.

42. Transform the plasmid: Transform 2 μL of 15 ng/μL concentrated plasmid containing MSP1D1 in a pET28a vector into 50 μL competent *E. coli* BL21 (DE3) cells by heat shock. Grow the cells in 250 μL LB media for 1 h. Spread 50–200 μL cell slurry onto an LB agar plate containing 50 μg/mL kanamycin. Place the plate in a 37°C incubator for 12–16 h.

Note: Transformed plates can be stored at 4°C for up to 2–3 weeks.

43. Scale up cell cultures: innoculate 10 mL LB media supplemented with 50 μg/mL kanamycin with a single colony and grow 12–14 h at 37°C with 200–250 rpm shaking. The next morning, use this culture to inoculate 1 L **Terrific Broth (TB) media** supplemented with 50 μg/mL kanamycin in a baffled flask. Allow the culture to grow at 37°C with 200–250 rpm shaking until reaching an optical density of 0.6–0.8 (typically about 5–6 h, but we recommend to check the cell density).
44. Induce protein expression: Induce protein expression by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to 1 mM final concentration, for 4 h at 37°C.

Note: For optimal MSP1D1 yield, do not induce protein expression for more than 4 h at 37°C. Extended postinduction growth results in a substantial decrease of the final yield.

45. Harvest cell cultures: Centrifuge the cell cultures at 4,000 × *g* for 30 min at 4°C. Resuspend the cell pellets in ~50 mL of ddH_2O using a serological pipette and transfer the cells into 50 mL plastic conical tubes. Spin down the cells at 3,200 × *g* for 15 min at 4°C. Remove the supernatant and freeze the pellets in liquid nitrogen. Store the pellets at −80°C.

Note: The expected yield is about 5–6 grams of wet cell pellet per liter of culture.

Note: The frozen cell pellets can be stored up to 10–12 months at −80°C.

46. Lyse the cells: If working with a frozen cell pellet, thaw the cells for ~30–40 min at 20°C–23°C. Resuspend the thawed cells in **MSP Lysis Buffer** at 5 mL/gram of cell pellet by vortexing. Add 100 μL of protease inhibitor solution, either from an in-house prepared stock or commercial source. Lyse the resuspended cells with a single pass through a cell disruptor (Pressure Biosciences) operating at 20,000 PSI, or lyse the cells via other standard techniques such as sonication.
47. Prepare lysate for chromatography: Spin down the lysed cells in an ultracentrifuge at 20,000 × *g* for 45 min at 4°C. Collect the supernatant and incubate it with Ni-NTA resin at 1 mL of resin per 1 L of cell culture equilibrated with **MSP Wash Buffer 2**, for 2 h rotating at 4°C.

Note: Prepare the Ni-NTA resin by washing it with 20 CVs of ddH_2O twice and 20 CVs of MSP Wash Buffer 2 once prior to adding the resin to the supernatant.

48. Remove impurities via IMAC: Load the incubated resin into an empty gravity flow column. Wash the column with 5 CVs of each of the following buffers in this order: **MSP Wash Buffer 2, MSP Wash Buffer 3, MSP Wash Buffer 1, and MSP Wash Buffer 4.**
49. Elute the protein: Elute MSP1D1 with 5 CVs of the **MSP Elution Buffer.** Collect the elution and measure the absorbance at 280 nm to estimate the protein concentration and yield.

Note: The extinction coefficient for MSP1D1 is 21, 000 M^{-1} cm^{-1} (with His-Tag) and 18,200 M^{-1} cm^{-1} (without His-Tag).

50. Prepare MSP1D1 for TEV cleavage: Exchange the purified MSP1D1 into the **MSP Desalting Buffer** using a prepacked Hiprep 26/10 Sephadex G-25 desalting column (Cytiva) and FPLC.

Note: The sample volume for Hiprep 26/10 Sephadex G-25 desalting column (column volume is 53 mL) is 15.9 mL. If sample volume is more than 15 mL, either concentrate the sample to 15.9 mL using Vivaspin®, 10,000 MWCO polyethersulfone concentrators or load the sample multiple times to the desalting column.

51. Remove the polyhistidine tag via TEV protease: Check the MSP1D1 concentration using a nanodrop device and estimate the total protein yield. Incubate MSP1D1 with TEV protease at a ratio of 1:100 (TEV:MSP) (w/w) 12–14 h at 4°C.

Note: While TEV can be purchased from commercial sources, it is much more cost effective to produce TEV in-house following published protocols (Cabrita et al., 2007).

52. Isolate cleaved MSP1D1: Add Ni-NTA resin to the solution containing cleaved MSP1D1 and TEV. Pipette the mixture in the gravity flow column and collect the flow-through fraction, which contains purified MSP1D1 lacking the polyhistidine tag.
53. Store purified MSP1D1: Exchange purified MSP1D1 into **MSP Dialysis Buffer (Storage Buffer)** by dialysis for 4 h using dialysis tubing with a 10 kDa MWCO (Thermo-Fisher Scientific). Collect a small 20 μL aliquot to check the quality of the purified protein by SDS-PAGE. Concentrate MSP1D1 to 1 mM. Aliquot into PCR or 1.7 mL tubes and freeze in liquid nitrogen. Store the frozen aliquots at −80°C.

Note: The purified MSP1D1 stocks can be kept for at least 6–8 months at −80°C.

Preparation of lipid stocks for nanodiscs assembly

Timing: 1 h

54. Prepare lipid stocks: Prepare 100 mM lipid stocks in cholate buffer by adding dry lipid powder to the **Nanodisc Cholate Buffer.** Vortex the solution and heat at 40°C–45°C until the powder is completely dissolved.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Monoclonal ANTI-FLAG® M2-Alkaline Phosphatase antibody produced in mouse	Sigma-Aldrich	Cat#A9469
Experimental models: Organisms/strains		
XL10-Gold ultracompetent cells	Agilent	Cat#200314
BL21 DE3 cells	Agilent	Cat#200132
P. pastoris: Bg12	BioGrammatics	Cat#PS004-01
Chemicals, peptides, and recombinant proteins		
n-Dodecyl-b-D-Maltopyranoside (DDM)	Anatrace	Cat#D310
Cholesteryl hemisuccinate (CHS)	Sigma-Aldrich	Cat#C6512
Adenosine 5′-triphosphate disodium salt hydrate (ATP)	Sigma-Aldrich	Cat#A2383
DPN I	New England Biolabs	Cat#R0176S
Biotin	Sigma-Aldrich	Cat#B4639
4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5] triazin-5-ylamino]ethyl)phenol (ZM241385)	Tocris Bioscience	Cat#1036
5′-N-Ethylcarboxamidoadenosine (NECA)	Tocris Bioscience	Cat#1691
Xanthine amine congener (XAC)	Tocris Bioscience	Cart#3200
4-[2-[[6-Amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl] benzenepropanoic acid hydrochloride (CGS21680)	Tocris Bioscience	Cat#1063
Theophylline	Sigma-Aldrich	Cat#T1633
[3H]4-[2-[7-amino-2-(2-furyl)-1,2,4-triazolo[1,5-a][1,3,5] triazin-5-yl-amino]ethylphenol ([3H]ZM241385)	American Radiolabeled Chemicals	Cat#ART0884-50
TALON Metal Affinity Resin	Gold bio	Cat#H-310-5
Ni-NTA Metal Affinity Resin	Sigma-Aldrich	Product#70666
1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC)	Avanti	SKU#850457
1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (POPS)	Avanti	SKU#840034
1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (sodium salt)	Avanti	SKU#870277
Cholic Acid, sodium salt	Anatrace	Cat#S1010S
Isopropyl β- d-1-thiogalactopyranoside (IPTG)	Goldbio	Cat#I2481C
Triton X-100	Sigma-Aldrich	Cat#T8787
SnakeSkin™ Dialysis Tubing, 10K MWCO, 22 mm	Thermo Fisher Scientific	Cat#68100
Bio-beads SM-2 Adsorbents	Bio-Rad	Cat#1523920
Glucose Oxidase	Sigma-Aldrich	Cat# G2133
Streptavidin	Thermo Fisher Scientific	Cat# S888
Cy3 maleimide,	GE Healthcare	PA23031
mPEG-SVA, MW 5,000	Laysan Bio	M-SVA
Biotin-PEG-SVA, MW 5,000	Laysan Bio	BIO-SVA
Catalase from bovine liver	Sigma-Aldrich	Cat# C100
N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)	Sigma-Aldrich	Cas#7365-45-9
TRIS Hydrochloride	Sigma-Aldrich	Cas#1185-53-1
Ethylenediaminetetraacetic acid	Sigma-Aldrich	Cas#60-00-4
Imidazole	Sigma-Aldrich	Cas#288-32-4
Glycerol	Sigma-Aldrich	Cas#56-81-5

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sodium Bicarbonate	Sigma-Aldrich	Cas#144-55-8
AEBSF	Gold-Bio	Cat#A-540-500
Leupeptin	Gold-Bio	Cat#L-010-5
E-64	Gold-Bio	Cat#E-064-5
Aprotinin	Gold-Bio	Cat#A-655-25
YNB w/o amino acids and ammonium sulfate	RPI Research Products	SKU#Y20060
Sodium phosphate monobasic anhydrous	Sigma-Aldrich	Cas#7558-80-7
Sodium phosphate dibasic anhydrous	Sigma-Aldrich	Cas#7558-79-4
Ammonium Sulfate	Sigma-Aldrich	Cas#7783-20-2
Magnesium Chloride	Sigma-Aldrich	Cas#7791-18-6
Adenosine 5′-triphosphate (ATP)	Sigma-Aldrich	Cas#34369-07-8
Methanol	Sigma-Aldrich	Cas#67-56-1
Yeast extract	Fisher Scientific	Cat#BP1422-2
Tryptone	Fisher Scientific	Cat#BP1421-500
Potassium phosphate dibasic (anhydrous)	RPI Research Products	Cas#7558-79-4
Potassium phosphate monobasic (anhydrous)	RPI Research Products	Cas#7558-80-7
$MgSO_4$	Sigma-Aldrich	Cas#7487-88-9
D-(+)-Glucose	Sigma-Aldrich	Cas#50-99-7
Deposited data		
Thermostabilized A_{2A}AR in complex with NECA	Lebon et al., 2011	PDB: 2YDV
A_{2A}AR in complex with NECA bound to an engineered G protein	Carpenter et al., 2016	PDB: 5G53
A_{2A}AR-BRIL bound to ZM241385	Liu et al., 2012	PDB: 4EIY
Oligonucleotides		
A_{2A}AR[A289C]		
Recombinant DNA		
Plasmid: human A_{2A}AR (1–316) in pPIC9K	Eddy et al., 2018	n/a
Plasmid: human A_{2A}AR[A289C]	This study	n/a
Software and algorithms		
GraphPad prism	GraphPad Software Inc.	https://www.graphpad.com
PyMOL	Pymol.org	https://pymol.org/2/
GROMACS	Gromacs.org	https://www.gromacs.org/
Maestro	Schrodinger	https://www.schrodinger.com/products/maestro
IDL	L3Harris Geospatial Solutions, Inc.	https://www.harrisgeospatial.com Version 8.7
HaMMy **smFRET data acquisition and analysis package**	McKinney et al. (2006)	http://ha.med.jhmi.edu/resources/ Version 4.0
Igor Pro	Wavemetrics, Inc.	https://www.wavemetrics.com Version 8.04
Single-molecule software	https://ha.med.jhmi.edu/	http://ha.med.jhmi.edu/resources/ Version 1.0
Other		
PD-10 column	Cytiva	Cat#17085101
Vivaspin® 6 ultrafiltration spin columns MWCO 30 kDa	Cytiva	Cat#28932361
MicroPulser Electroporator	Bio-Rad	Cat# #1652100
Incubator shaker	Eppendorf	Cat#M1282-0010
Fernbach Flask	VWR	https://us.vwr.com/store/product/4589333/pyrex-fernbach-culture-flasks-corning
Beckman Coulter Optima XE-90 Ultracentrifuge	Beckman Coulter	https://www.beckman.com/centrifuges/ultracentrifuges/optima-xe/a94471
Large Centrifuge JXN 26	Beckman Coulter	https://www.beckman.com/centrifuges/high-speed/avanti-jxn-26#
Eppendorf Centrifuge 5180R	Eppendorf	Cat#022628089
Eppendorf Centrifuge5424R	Eppendorf	

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fast protein liquid chromatography (FPLC) system – AKTA Pure	Cytiva	https://www.cytivalifesciences.com/en/us/shop/chromatography/chromatography-systems/akta-pure-p-05844
High performance liquid chromatography (HPLC) system – 1260 Infinity II	Agilent Technologies	https://www.agilent.com/en/product/liquid-chromatography/hplc-systems/analytical-hplc-systems/1260-infinity-ii-lc-system
Dremel 220-01 drill	Dremel	https://www.dremel.com/us/en/p/220-01-26150220aa
Diamond Drills (0.75 mm)	Kingsley North, Inc	SKU#3099425_CFG
Bunsen burner	n/a	n/a
Double-side tape	Scotch, 3 M	n/a
A customized TIRF set-up using Inverted IX73 microscope frame	Olympus, water-immersion	n/a
EMCCD camera	Andor Technology	n/a
TIRF stage	TIRF Labs	n/a
532 nm laser	CrystaLaser	n/a
Sonication bath	Fisherbrand	Cat#CPX2800
Slides holder	Teflon	n/a
Micro slide staining rack	Steel	n/a
Glass coplin jars	LabScientific	https://labscientific.com/glass-coplin-staining-jar-histology
1″ × 3″ × 1 mm thick quartz slides	G. Finkenbeiner Inc.	https://finkenbeiner.com/quartzslides
Superslip cover slips	Fisherbrand	Cat#254588

MATERIALS AND EQUIPMENT

Terrific Broth (TB) media

Reagent	Final concentration	Amount
Yeast extract	24 g/L	24 g
Tryptone	12 g/L	12 g
Glycerol	4 mL/L	4 mL
Potassium phosphate dibasic (anhydrous)	9.4 g/L	9.4 g
Potassium phosphate monobasic (anhydrous)	2.2 g/L	2.2 g
D-(+)-Glucose	2 g/L	2 g
$MgSO_4$	0.6 g/L	0.6 g
ddH_2O	n/a	1000 mL
Total	**n/a**	1000 mL

Add the D-(+)-glucose and $MgSO_4$ after the rest of the media has been sterilized (by autoclaving) and cooled to 25°C.

Protease Inhibitor Stock

Reagent	Final concentration	Amount
AEBSF	500 μM	5 g
E-64	1 μM	25 mg
Leupeptin	1 μM	100 mg
Aprotinin	150 nM	100 mg
ddH_2O	n/a	42 mL
Total	**n/a**	**42 mL**

Protease Inhibitor stocks can be stored at −80°C for 1–1.5 years.

Gel Loading Buffer (2×)		
Reagent	Final concentration	Amount
Tris-HCl (pH 6.8) (1 M)	125 mM	6.24 mL
SDS (10% v/v)	4% (v/v)	20 mL
Glycerol	20% (v/v)	10 mL
Bromophenol blue	0.02% (w/v)	0.01 mg
ddH_2O	n/a	13.76 mL
Total	**n/a**	**50 mL**
Adjust pH of the buffer to be at pH 6.8.		

Buffers required for western-blot analysis

Blocking Solution		
Reagent	Final concentration	Amount
Tween	0.05% (v/v)	12.5 µL
PBS (1×)	n/a	2.5 mL
Dry Milk	5% (w/v)	1250 mg
ddH_2O	n/a	22.5 mL
Total	**n/a**	**25 mL**
Prepare buffer fresh, Prepare 1× PBS buffer from PBS tablets.		

Washing Solution		
Reagent	Final concentration	Amount
Tween	0.05% (v/v)	12.5 µL
PBS (1×)	n/a	2.5 mL
ddH_2O	n/a	22.5 mL
Total	**n/a**	**25 mL**
Prepare buffer fresh, Prepare 1× PBS buffer from PBS tablets.		

Developing Solution		
Reagent	Final concentration	Amount
Tween	0.05% (v/v)	10 µL
PBS (1×)	n/a	2 mL
M2 anti-FLAG Conjugated Antibody	0.05% (v/v)	10 µL
ddH_2O	n/a	18 mL
Total	**n/a**	**20 mL**
Prepare buffer fresh, Prepare 1× PBS buffer from PBS tablets.		

Buffers required for MSP1D1 purification

MSP Lysis Buffer		
Reagent	Final concentration	Amount
Tris-HCl (pH 8.0) (1 M)	50 mM	25 mL
NaCl (5 M)	500 mM	50 mL
Triton X-100	1% (v/v)	5 mL
EDTA (0.5 M)	1 mM	1 mL
ddH_2O	n/a	419 mL
Total	**n/a**	**500 mL**
Store at 4°C for 3–4 weeks.		

MSP Wash Buffer 1		
Reagent	Final concentration	Amount
Tris-HCl (pH 8.0) (1 M)	50 mM	5 mL
NaCl (5 M)	500 mM	10 mL
ddH_2O	n/a	85 mL
Total	**n/a**	**100 mL**
Store at 4°C for 4–8 weeks.		

MSP Wash Buffer 2		
Reagent	Final concentration	Amount
Tris-HCl (pH 8.0) (1 M)	50 mM	5 mL
NaCl (5 M)	500 mM	10 mL
Triton X-100	1% (v/v)	1 mL
ddH_2O	n/a	84 mL
Total	**n/a**	**100 mL**
Store at 4°C for 3–4 weeks.		

MSP Wash Buffer 3		
Reagent	Final concentration	Amount
Tris-HCl (pH 8.0) (1 M)	50 mM	5 mL
NaCl (5 M)	500 mM	10 mL
Sodium Cholate (200 mM)	50 mM	25 mL
ddH_2O	n/a	60 mL
Total	**n/a**	**100 mL**
Store at 4°C for 1–2 weeks.		

MSP Wash Buffer 4		
Reagent	Final concentration	Amount
Tris-HCl (pH 8.0) (1 M)	50 mM	5 mL
NaCl (5 M)	500 mM	10 mL
Imidazole (pH 7.5) (1 M)	20 mM	2 mL
ddH_2O	n/a	83 mL
Total	**n/a**	**100 mL**
Store at 4°C for 3–4 weeks. Adjust the pH of the 1 M imidazole stock to pH 7.5 with a base such as NaOH and store in the dark for up to 2 months.		

MSP Elution Buffer		
Reagent	Final concentration	Amount
Tris-HCl (pH 8.0) (1 M)	50 mM	5 mL
NaCl (5 M)	500 mM	10 mL
Imidazole (pH 7.5) (1 M)	500 mM	50 mL
ddH_2O	n/a	35 mL
Total	**n/a**	**100 mL**
Store at 4°C for 3–4 weeks. Adjust the pH of the 1 M imidazole stock to pH 7.5 with a base such as NaOH and store in the dark for up to 2 months.		

MSP Desalting Buffer		
Reagent	Final concentration	Amount
Tris-HCl (pH 8.0) (1 M)	50 mM	25 mL
NaCl (5 M)	20 mM	4 mL
EDTA (0.5 M)	0.5 mM	0.5 mL
ddH_2O	n/a	470.5 mL
Total	**n/a**	**500 mL**
Store at 4°C for 4–8 weeks.		

MSP Dialysis Buffer (Storage Buffer)		
Reagent	Final concentration	Amount
Tris-HCl (pH 8.0) (1 M)	20 mM	40 mL
NaCl (5 M)	100 mM	40 mL
EDTA (0.5 M)	0.5 mM	2 mL
ddH_2O	n/a	1918 mL
Total	**n/a**	**2000 mL**
Store at 4°C for 4–8 weeks.		

Buffers for $A_{2A}AR$ expression

BMGY		
Reagent	Final concentration	Amount
YNB w/o Amino acids and ammonium sulfate	n/a	4.25 g
Sodium Phosphate Monobasic Anhydrous	100 mM	11.7 g
Ammonium Sulfate	38 mM	5 g
Glycerol	2% (v/v)	20 mL
Biotin (0.02% w/v)	0.00005% (w/v)	2.5 mL
Sodium Phosphate Dibasic Anhydrous	50 mM	7.5 g
ddH_2O	n/a	987.5 mL
Total	**n/a**	**1000 mL**
Store at 20°C–23°C for 2–3 days.		

BMMY		
Reagent	Final concentration	Amount
YNB w/o Amino acids and ammonium sulfate	n/a	4.25 g
Sodium Phosphate Monobasic Anhydrous	100 mM	11.7 g
Ammonium Sulphate	38 mM	5 g
Biotin (0.02% w/v)	0.00005% (w/v)	2.5 mL
Sodium Phosphate Dibasic Anhydrous	50 mM	7.5 g
ddH_2O	n/a	987.5 mL
Total	**n/a**	**1000 mL**
Store at 20°C–23°C for 2–3 days.		

Buffers for $A_{2A}AR$ purification

$A_{2A}AR$ Lysis Buffer		
Reagent	Final concentration	Amount
Sodium Phosphate Monobasic Anhydrous	50 mM	4.62 g
Sodium Phosphate Dibasic Anhydrous	50 mM	8.74 g

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Continued		
Reagent	Final concentration	Amount
NaCl	10 mM	11.6 g
Glycerol	5% (w/v)	100 g
ddH_2O	n/a	2000 mL
Total	**n/a**	**2000 mL**
Store at 4°C for 3–4 weeks.		

A_{2A}AR High Salt Buffer		
Reagent	Final concentration	Amount
HEPES (pH 7) (1 M)	10 mM	20 mL
KCl (1 M)	10 mM	20 mL
$MgCl_2$ (1 M)	20 mM	40 mL
NaCl	1000 mM	116.8 g
ddH_2O	n/a	1920 mL
Total	**n/a**	**2000 mL**
Store at 4°C for 3–4 weeks.		

A_{2A}AR Solubilization Buffer		
Reagent	Final concentration	Amount
HEPES (pH 7) (1 M)	100 mM	20 mL
NaCl (5 M)	500 mM	20 mL
DDM-CHS (10%–1%) (w/w)	1%–0.1%	20 mL
ddH_2O	n/a	140 mL
Total	**n/a**	**200 mL**
Prepare fresh buffer.		

A_{2A}AR Wash Buffer 1		
Reagent	Final concentration	Amount
HEPES (pH 7) (1 M)	50 mM	5 mL
Imidazole (pH 7.5) (3 M)	30 mM	1 mL
$MgCl_2$ (1 M)	10 mM	1 mL
NaCl (5 M)	500 mM	10 mL
ATP	11 mM	564.6 mg
DDM-CHS (10%–1%) (w/w)	0.05%–0.005%	0.5 mL
ddH_2O	n/a	82 mL
Total	**n/a**	**100 mL**
Prepare fresh buffer.		

A_{2A}AR Wash Buffer 2		
Reagent	Final concentration	Amount
HEPES (pH 7) (1 M)	50 mM	5 mL
Imidazole (pH 7.5) (3 M)	30 mM	1 mL
NaCl (5 M)	250 mM	5 mL
Glycerol	5% (w/v)	5 g
DDM-CHS (10%–1%) (w/w)	0.05%–0.005%	0.5 mL
Ligand	n/a	n/a
ddH_2O	n/a	88 mL
Total	**n/a**	**100 mL**
Saturating amount of ligand will be calculated on the bases of ligand affinity value. Prepare fresh buffer.		

A_{2A}AR Wash Buffer 3		
Reagent	Final concentration	Amount
HEPES (pH 7) (1 M)	50 mM	5 mL
Imidazole (pH 7.5) (3 M)	30 mM	1 mL
NaCl (5 M)	250 mM	5 mL
Glycerol	5% (w/v)	5 g
DDM-CHS (10%–1%) (w/w)	0.05%–0.005%	0.5 mL
Ligand	n/a	n/a
ddH_2O	n/a	88 mL
Total	**n/a**	**100 mL**

Saturating amount of ligand will be calculated on the bases of ligand affinity value. Prepare fresh buffer.
Set the pH of the 3 M imidazole stock to pH 7.5 with a strong base such as NaOH and store in the dark for up to 2 months.

A_{2A}AR Elution Buffer		
Reagent	Final concentration	Amount
HEPES (pH 7) (1 M)	25 mM	1.25 mL
Imidazole (pH 7.5) (3 M)	300 mM	2.5 mL
NaCl (5 M)	250 mM	1.25 mL
Glycerol	5% (w/v)	1.25 g
DDM-CHS (10%–1%) (w/w)	0.05%–0.005%	0.125 mL
Ligand	n/a	n/a
ddH_2O	n/a	19 mL
Total	**n/a**	**25 mL**

Saturating amount of ligand will be calculated on the bases of ligand affinity value. Prepare fresh buffer.
Set the pH of the 3 M imidazole stock to pH 7.5 and store in dark at 4°C for upto 2 months.

A_{2A}AR Desalting Buffer		
Reagent	Final concentration	Amount
HEPES (pH 7) (1 M)	20 mM	0.5 mL
NaCl (5 M)	150 mM	0.75 mL
DDM-CHS (10%–1%) (w/w)	0.05%–0.005%	0.125 mL
Ligand	n/a	n/a
ddH_2O	n/a	23.625 mL
Total	**n/a**	**25 mL**

Saturating amount of ligand will be calculated on the bases of ligand affinity value. Prepare fresh buffer.

Buffers required for nanodiscs preparation

Nanodiscs Cholate Buffer		
Reagent	Final concentration	Amount
Tris-HCl (pH 8) (1 M)	25 mM	0.125 mL
Sodium Cholate	200 mM	0.431 g
NaCl (5 M)	100 mM	0.1 mL
ddH_2O	n/a	4.775 mL
Total	**n/a**	**5 mL**

Prepare fresh buffer. HEPES can be used instead of Tris-HCL if needed.

Nanodiscs Buffer		
Reagent	Final concentration	Amount
HEPES (pH 7) (1 M)	25 mM	0.125 mL
EDTA (0.5 M)	0.5 mM	0.5 μL
NaCl (5 M)	100 mM	0.1 mL
ddH_2O	n/a	4.775 mL
Total	**n/a**	**5 mL**
Prepare fresh buffer.		

Nanodiscs Wash Buffer		
Reagent	Final concentration	Amount
HEPES (pH 7) (1 M)	50 mM	0.5 mL
Imidazole (pH 7.5) (3 M)	10 mM	0.033 mL
NaCl (5 M)	150 mM	0.3 mL
ddH_2O	n/a	9.5 mL
Total	**n/a**	**10 mL**
Prepare fresh buffer. Adjust the pH of the 3 M imidazole stock to 7.5 and store in the dark for up to 2 months.		

Nanodiscs Elution Buffer		
Reagent	Final concentration	Amount
HEPES (pH 7) (1 M)	50 mM	0.5 mL
Imidazole (pH 7.5) (3 M)	300 mM	1 mL
NaCl (5 M)	150 mM	0.3 mL
ddH_2O	n/a	9.5 mL
Total	**n/a**	**10 mL**
Prepare this buffer fresh every time. Adjust the pH of the 3 M imidazole stock to 7.5 and store in the dark for up to 2 months.		

Nanodiscs Desalting Buffer		
Reagent	Final concentration	Amount
HEPES (pH 7) (1 M)	25 mM	1.25 mL
NaCl (5 M)	75 mM	0.75 mL
ddH_2O	n/a	48 mL
Total	**n/a**	**50 mL**
Prepare fresh buffer.		

Nanodiscs SEC Buffer		
Reagent	Final concentration	Amount
HEPES (pH 7) (1 M)	25 mM	12.5 mL
NaCl (5 M)	150 mM	15 mL
ddH_2O	n/a	472.5 mL
Total	**n/a**	**500 mL**
Prepare fresh buffer.		

Buffers required for A_{2A}AR-nanodisc single-molecule TIRF experiments

Piranha Solution		
Reagent	Final concentration	Amount
Concentrated ammonium hydroxide	20%	40 mL
Concentrated hydrogen peroxide	20%	40 mL
ddH_2O	n/a	120 mL
Total	**1 M**	**200 mL**

Aminosilanization Solution		
Reagent	Final concentration	Amount
(3-Aminopropyl)triethoxysilane (3-APTES)	3%	9 mL
Acetic acid	5%	15 mL
Methanol	n/a	276 mL
Total	**n/a**	**300 mL**

Imaging Buffer		
Reagent	Final concentration	Amount
1 M HEPES, pH 7.5	25 mM	1.25 mL
1 M NaCl	75 mM	3.75 mL
2 mM Trolox solution	n/a	45 mL
Total	**n/a**	**50 mL**

STEP-BY-STEP METHOD DETAILS

A_{2A}AR ^{19}F-Trifluoroethanethiol (TET) labeling for NMR experiments

Timing: 2 days

This section describes the steps to label A_{2A}AR with ^{19}F-Trifluoroethanethiol (TET) during purification of the receptor in detergent micelles, which will be subsequently used in the assembly of lipid nanodisc samples (Figure 5A).

Note: The following steps are done after step 33 in the "purification of A_{2A}AR for nanodisc assembly" sub-section in the "before you begin" section. For producing samples for single-molecule fluorescence, skip the following steps and proceed to the section nanodisc assembly and Cy3 labeling for single-molecule fluorescence.

1. Treat membranes with Aldrithiol: Resuspend the membranes in a glass tissue homogenizer with 100 mL **A_{2A}AR High Salt Buffer** containing 4 mM theophylline. Completely resuspend the membranes using 30 or more strokes, resulting in a homogeneous solution. Add 1 mM aldrithiol (22 mg/liter of cell culture, MW:220.31) and 100 µL of **in-house Protease Inhibitor** to the resuspended membranes. Resuspend the reagents in the membrane mixture, distribute among 50 mL conical tubes and rotate at 4°C for 1 h.
2. Treat membranes with trifluoroethanethiol (TET): Pellet the resuspended membranes in an ultracentrifuge at 200,000 × *g* for 30 min at 4°C. Discard the supernatant and resuspend the membrane pellet in 100 mL **A_{2A}AR High salt buffer.** Add TET to a final concentration of 1 mM/ liter of cell culture. Incubate the sample at 4°C for 1 h. Pellet the membranes in an ultracentrifuge at 200,000 × *g* for 30 min and discard the supernatant.

Note: TET is toxic. Application of TET and resuspension of TET-containing membrane mixtures must be done in a chemical hood and with appropriate personal protective equipment.

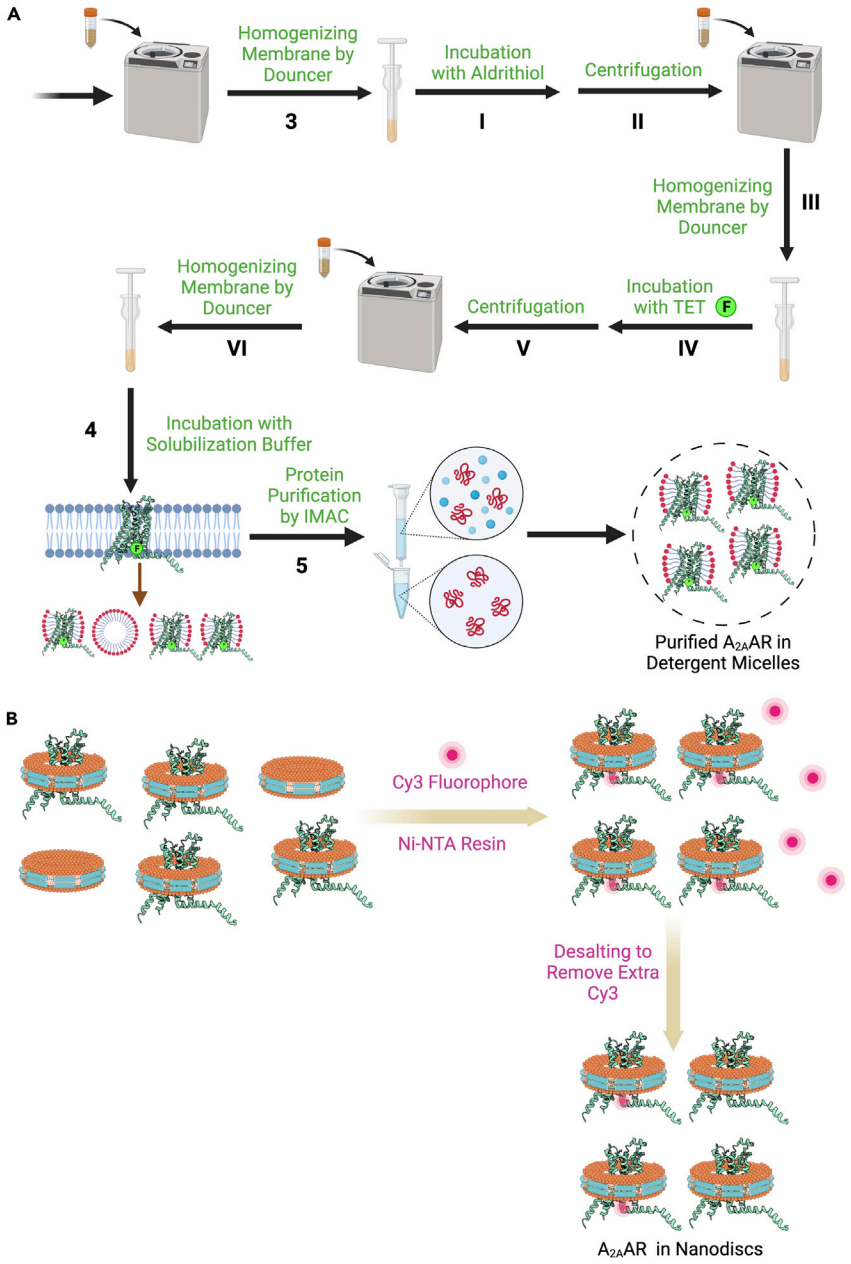

Figure 5. Work flow of ^{19}F-TET labeling of $A_{2A}AR$ in *P. pastoris* membranes and Cy3 fluorophore labeling of $A_{2A}AR$ in nanodiscs

(A) Individual steps for in-membrane chemical modification of $A_{2A}AR$ with ^{19}F-trifluoroethanethiol (TET) for NMR experiments. Individual steps for incorporation of ^{19}F-TET are indicated with roman numerals and occur between steps '3' and '4' in Figure 3A. Note that steps I-VI would be skipped for preparation of fluorescently-labeled $A_{2A}AR$ for SMF experiments.

(B) Individual steps for incorporation of a Cy3 cysteine-reactive fluorophore for single-molecule fluorescence experiments. Figure created with BioRender.com.

> ***Note:*** Deactivate solutions containing TET with 20% bleach and thoroughly wash all apparatus from this step using standard lab detergents and water as soon as you have finished using them.

3. Continue to steps 46–53 described in the section "purification of $A_{2A}AR$ for nanodisc assembly."

Nanodisc assembly and Cy3 labeling for single-molecule fluorescence

Timing: 3 days

This section describes the stepwise process to assemble nanodiscs containing $A_{2A}AR$ and labeling the receptor with the Cy3 dye for single molecule fluorescence experiments.

4. Prepare lipids: Prepare 100 mM stocks of lipids in **Nanodisc Cholate Buffer.**

   Note: It is critical that the lipids completely dissolve before proceeding. Some lipids require gentle heating and shaking to completely dissolve. Heat solutions to 40°C–50ºC for up to 1 h with gentle shaking to ensure complete dissolution. For more information about the phase transition temperature for lipids refer Avanti website (https://avantilipids.com/tech-support/physical-properties/phase-transition-temps).

5. Prepare for nanodisc assembly: Calculate amounts of receptor, lipids and MSP1D1 required to form nanodiscs based on the reported optimized molar ratio of 1:5:250 (receptor:MSP1D1:lipids). An example calculation is provided below.

Component (stock concentration)	Volume	Final concentration
Receptor (53 μM)	500 μL	26.5 μM
MSP1D1 (1 mM)	132.5 μL	132.5 μM
Lipids (100 mM)	66.2 μL	6.62 mM
Nanodisc Cholate Buffer (200 mM)	33.8 μL	20 mM
Nanodisc Buffer	267.5 μL	n/a
Total	1 mL	n/a

   Note: The optimal ratio depends on the size of the membrane protein, particular MSP construct and lipids and may be different for other membrane proteins or other MSPs.

6. Initiate nanodisc assembly (Figure 3): Add all the components to a microcentrifuge tube in the following order: **Nanodisc Buffer, Nanodisc Cholate Buffer,** lipids, MSPD1 and $A_{2A}AR$. Incubate the assembly mixture for 1–2 h with gentle rotation at 4°C.

   Note: The incubation temperature should be near the phase-transition temperature of the lipids. For mixtures of lipids, incubate the solution near the phase transition temperature of the largest molar fraction of lipids in the sample.

7. Remove detergent: Add 0.5–1 g Bio-Beads/mL of nanodisc assembly mixture. Incubate the sample for 12–14 h at 4°C with gentle rocking or rotation.
8. Remove the Bio-Beads: Use a syringe with a 20-gauge needle to collect the soluble fraction and apply to a fresh tube. Wash the Bio-Beads once with an equal volume of **Nanodiscs Buffer** and combine with the first collected aliquot.
9. Prepare Ni-NTA by washing it with 5 CVs of ddH_2O. Add the Ni-NTA resin to the pooled sample and incubate it at 4°C for 12–14 h. For every 1 mL of assembled nanodisc solution add 250 μL Ni-NTA resin.

   Note: For single-molecule fluorescence, the following steps must be performed in dark conditions as exposure of light will bleach the fluorophore. For NMR sample preparation, skip to step 11. NMR samples are not sensitive to light.

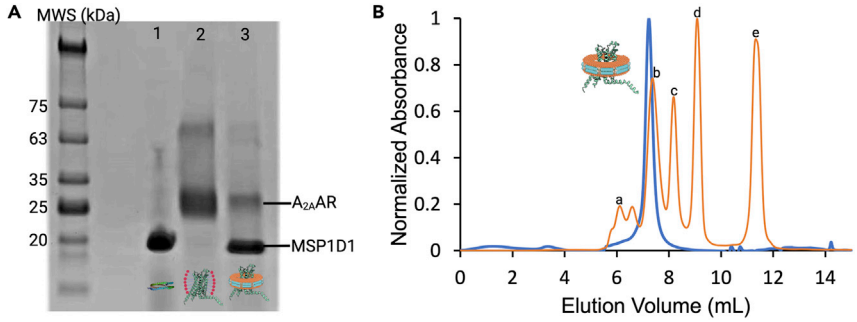

Figure 6. SDS-PAGE and SEC characterization of lipid nanodiscs containing $A_{2A}AR$

(A) Representative SDS-PAGE gel of assembled nanodisc components and isolated nanodiscs containing $A_{2A}AR$. Lanes labeled 1, 2, and 3 correspond to samples containing purified MSP1D1, $A_{2A}AR$ in detergent micelles, and isolated nanodiscs containing $A_{2A}AR$, respectively. The bands corresponding to $A_{2A}AR$ and MSP1D1 are annotated in lane 3. The lane labeled "MWS" corresponds to molecular weight standards. Note that the higher molecular weight band in lanes 2 and 3 (~63 kDa) is an artificial GPCR dimer created by SDS.

(B) Representative analytical size exclusion chromatogram of purified nanodiscs containing $A_{2A}AR$, shows a single peak (blue) consistent with a homogenous preparation. The superimposed chromatogram in orange was obtained with MWS (Bio-Rad). The peaks correspond to the following molecular weights: a - 670 kDa, b - 158 kDa, c - 44 kDa, d - 17 kDa, e - 1.3 kDa.

Figure created with BioRender.com.

10. Add the fluorophore (Figure 5B): Add the Cy3 fluorescent dye at a 10× fold molar excess of receptor to the sample. Incubate at 4°C for 2–3 h with rotation.
11. Remove empty nanodiscs: Transfer the resin to a gravity flow column. Wash the resin with 3 CVs of **Nanodisc Wash Buffer.** Elute the nanodiscs containing $A_{2A}AR$ using **Nanodiscs Elution Buffer** in 10 different 0.5 CV fractions. Measure the protein absorbance in each aliquot and pool the 3–4 aliquots with the highest protein concentration.
12. Exchange the sample into the final buffer: Using a PD-10 column, follow the manufacturer's protocol to exchange the sample into the **Nanodiscs Desalting Buffer.** https://cytiva-delivery.sitecorecontenthub.cloud/api/public/content/digi-11531-pdf.
13. Characterize the sample: Use Nanofilm SEC-250, 5 μm, 250 Å, 7.8 × 300 mm (Sepax Technologies) analytical size exclusion chromatography to check the monodispersity and homogeneity of samples (Figure 6) from step 12.

NMR sample preparation

Timing: 3–4 h

This section describes the steps to prepare the final nanodisc sample in the correct concentration required for ^{19}F-NMR experiments.

14. Concentrate the sample: Assuming the sample will be transferred to a Shigemi susceptibility-matched NMR tube, concentrate the NMR sample to a final volume of ~280 μL. Concentrate the sample by applying 300–400 × *g* for a duration of 20–25 min in a refrigerated tabletop centrifuge at 4°C using a 30,000 MWCO polyethersulfone concentrator (Vivaspin®). Carefully resuspend the sample and repeat until reaching the desired concentration.

Note: Do not exceed 300–400 × *g* during this step as this can cause protein aggregation.

15. Add 20 μL 99.8% D_2O and gently but thoroughly mix the sample with manual pipetting. The D_2O is required for the NMR spectrometer lock and is a standard component of NMR samples.

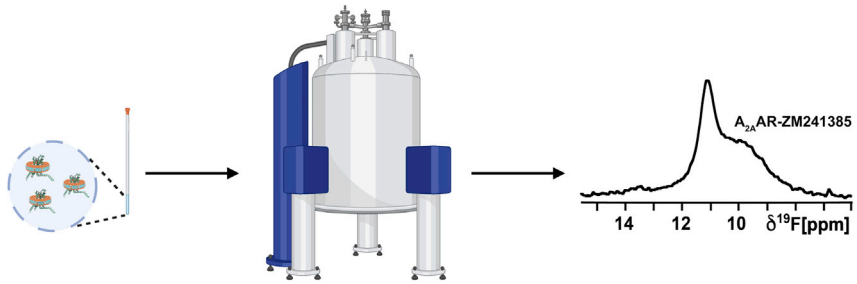

Figure 7. Representative ^{19}F-NMR data of $A_{2A}AR$

A representative 1-dimensional ^{19}F-NMR spectrum is shown of lipid nanodiscs containing $A_{2A}AR[A289C^{TET}]$ in complex with the antagonist ZM241385. Figure created with BioRender.com.

16. Transfer to an NMR tube: Transfer the sample to a Shigemi susceptibility-matched NMR tube using a standard manual 200 μL pipette.
17. Setup the spectrometer for ^{19}F detection according to your institution's approved procedures. Record an NMR spectrum (Figure 7) using the standard 'zg' one-pulse program provided by Bruker. Additional experimental details and typical acquisition parameters for ^{19}F-NMR with $A_{2A}AR$ have been described in greater detail (Sušac et al., 2018).

Preparation of microfluidic channels for single-molecule fluorescence experiments

Timing: 15 min

18. Prepare microfluidic channels: Drill two holes into 1″ × 3″ × 1 mm quartz slides by using a hand drill with 0.75 mm drill bits. The distance between the two holes is 4.5 cm (Figure 8A).

Preparing quartz slides

Timing: 2 h

This section describes steps used to clean slides that will be used for single-molecule fluorescence experiments.

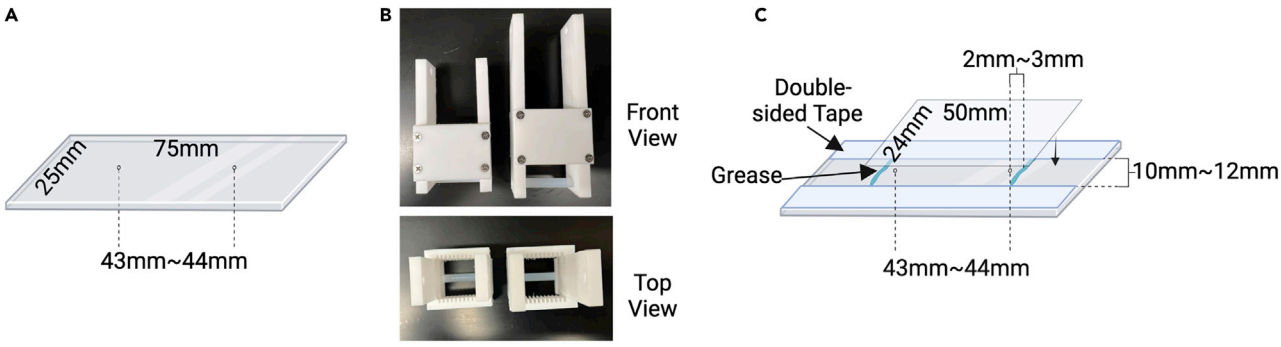

Figure 8. Microscope slide cleaning, PEG passivation, and chamber assembly for single-molecule fluorescence experiments

(A) Diagram of a typical microscope slide used for SMF TIRF experiments with relevant dimensions. Drill two holes on the slide to prepare a microfluidic chamber for flowing solutions containing protein samples and buffers.

(B) Custom-made Teflon slide holder for slide cleaning and passivation.

(C) Preparation of the channel for sample immobilization using double-sided tape and grease. Relevant dimensions are indicated.

Figure created with BioRender.com.

19. Boil recycled slides: If recycling slides from previous experiments, boil the quartz slides for 15 min to remove debris from previous experiments. If working with new slides, skip to the next step.

Note: From our experience, slides can be used at least 4–5 times before needing to replace them.

20. Sonicate the slides: Place the slides in a slide holder (e.g., prepared in house from Teflon or glass coplin staining jars, Figure 8B). Insert the slides and holder into new beaker, add 20% detergent solution (Contrad 70) and sonicate for 20 min. Discard the detergent solution, rinse with Milli-Q water 3 times, and sonicate for 40 min in Milli-Q water.
21. Thoroughly clean the slides: Boil slides in a beaker containing **Piranha Solution** for 15 min, making sure slides are fully immersed in the solution. Discard the solution to a labeled segregated container after use for proper disposal.

Note: Ammonium hydroxide and hydrogen peroxide are irritants. Use all recommended safety precautions, including appropriate personal protective equipment and working in a fume hood.

22. Rinse and dry the slides: Thoroughly rinse the slides with Milli-Q water to remove any remaining piranha solution. Collect the washes and discard into a labeled segregated container. Dry the slides by carefully applying above a Bunsen burner. Cool the slides in a slide staining rack and place them into the slide holder until needed.

Surface activation of slides and coverslips

Timing: 2 h

This section describes steps used to chemically treat the cleaned quartz slides and coverslips to prepare them for subsequent SMF experiments.

23. Activate the slides and coverslips: Immerse quartz slides and 50 mm × 24 mm glass coverslips in 1 M potassium hydroxide solution and sonicate for 20 min.

Note: potassium hydroxide is a strong base and caustic. Take precautions including wearing appropriate personal protective equipment while handling it.

24. Rinse the slides: Discard the potassium hydroxide solution. Rinse slides well with ddH_2O, discard, and then sonicate with fresh ddH_2O for 40 min.
25. Rinse and dry slides: Discard the water and fill jars with methanol. Sonicate for 40 min. Discard the methanol and rinse the slides at least 3 times with ddH_2O. Dry the slides and coverslips by flushing with nitrogen gas.

Slide surface aminosilanization

Timing: 30 min

This section describes the process to silanize the quartz slides, which reduces the adherence of proteins directly to the slide.

26. Apply **Aminosilanization Solution**: Carefully prepare 300 mL **Aminosilanization Solution (2%–3% APTES)** in a clean and dry beaker. Add the solution into a beaker containing the slide holder

containing slides and coverslips and incubate at 20°C–23°C for 10 min. Sonicate the slides and coverslips with the solution for 2 min and incubate again for 10 min.

Note: 3-APTES is highly toxic and carcinogenic. Do this step in a fume hood and use appropriate personal protective equipment.

27. Rinse and dry the slides: Discard the **Aminosilanization Solution** into a proper labeled segregated container. Rinse the slides thoroughly with 100% methanol and discard. Then rinse the slides thoroughly with ddH_2O and discard. Rinse the slides one final time with methanol and discard.
28. Dry the slides: Thoroughly dry the slides by applying a stream of nitrogen gas.

Slide surface PEGylation

Timing: 30 min preparation + 10–12 h incubation, approximately 12–13 h in total

This section details steps for PEGylating the quarts slides.

29. Prepare solution: Prepare fresh 100 mM sodium bicarbonate solution and filter with a Millex-GP 0.22-micron filter.
30. Prepare PEG solution: Dissolve 80 mg of mPEG-SVA and 5 mg of Biotin-PEG-SVA by gently adding 400 μL sodium bicarbonate buffer and vortexing until the mixture becomes transparent. Centrifuge the solution for one minute at 10,000 × *g* to remove bubbles. This amount will be enough to PEGylate 5 slides.
31. Apply PEG solution on the slides: Add 90 μL of PEG solution to each quartz slide. Gently apply a coverslip on the top of the PEG solution on the slide, avoiding the formation of bubbles on the slide.
32. Incubate slides in the dark 12–14 h.

Note: To prevent desiccation-induced adhesion of the quartz slides to the coverslips, the slides need to be incubated in a closed container that maintains a humid environment. We use empty clean pipet tip boxes with water at the bottom.

Cleaning and storage

Timing: 1 h

This section describes steps for preparing the quartz slides for storing until they are used for SMF experiments.

33. Outline the PEG surface with a marker. Then carefully separate the coverslips from slides.
34. Rinse and store slides: Rinse both slides and coverslips thoroughly with ddH_2O, dry them with nitrogen and store in a 50 mL centrifuge tube with flushed nitrogen. Seal the tube with parafilm and stored at −20°C.

Note: Keep the nitrogen gas pressure lower than 10 pounds per square inch (PSI) while drying the PEG surface.

Note: The slides should be stable for 3–4 weeks. Additional details regarding slide cleaning and passivation can be found also in earlier work (Lamichhane et al., 2010).

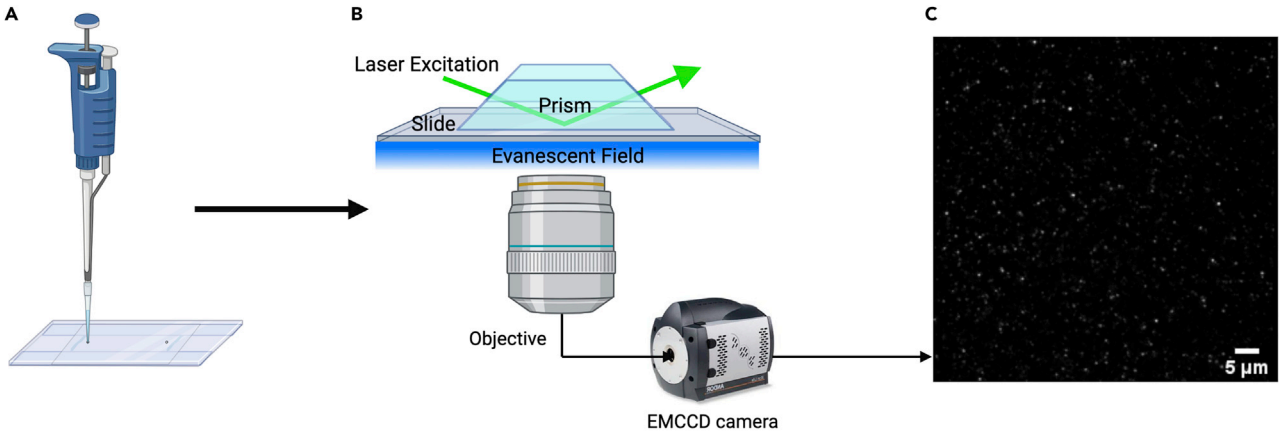

Figure 9. Single-molecule fluorescence sample immobilization and data acquisition workflow

(A) Sample loading diagram. Use a manual pipette to directly inject samples into the microfluidic chamber.

(B) Schematic diagram of the prism based TIRF microscope for SMF data collection. The immobilized sample on the microscope slide is illuminated by an evanescent wave of a 532 nm laser beam. An EMCCD camera records a molecular movie of the observed fluorescence signals.

(C) A representative TIRF field-of-view image from an immobilized sample of A_{2A}AR in lipid nanodiscs. Figure created with BioRender.com.

A_{2A}AR-nanodisc complex immobilization and addition of ligands

Timing: 15–20 min

This section describes the process by which the nanodisc sample is fixed to the quartz slide and ligands are added to the sample (Figure 9A).

35. Warm up slides: Take out slide from freezer storage and allow it to equilibrate to 20°C–23°C.
36. Prepare sample chamber: Use double-sided tape and grease to prepare the sample chamber on a clean, PEGylated quartz surface marked with a marker as in step 33 of "step-by-step method details".
37. Add streptavidin: Pipette 50 μL of 0.2 mg/mL streptavidin (Thermo Fisher) into the sample chamber through the hole on the quartz slide and incubate for 5 min to let streptavidin bind to the Biotin-PEG on the slide.
38. Wash the chamber: Wash out unbound streptavidin by pipetting 120 μL of imaging buffer into the sample chamber. The imaging buffer should contain a saturating concentration of ligand, or no ligand for the apo sample.
39. Add the A_{2A}AR-nanodisc complex into the chamber, approximately 120 μL at a concentration of 50 pM. Incubate for 5–10 min to immobilize nanodisc sample contains 5% biotin-PE on the streptavidin coated quartz slide surface. Rinse the chamber with 120 μL **Imaging Buffer** to remove uncombined sample.
40. Add reagents to increase dye stability: Apply an additional 120 μL imaging buffer containing glucose oxidase and a catalase oxygen scavenging system (OSS) to the chamber to increase the dye's photostability. The buffer should also contain either a saturating concentration of the same ligand used in step 38 or no ligand. The glucose oxidase and a catalase oxygen scavenging system contains 10% w/v glucose, 50 μg/mL glucose oxidase and 10 μg/mL bovine catalase prepared by adding the reagents to the imaging buffer.

Single-molecule data acquisition

Timing: 40 min

This section outlines the steps for handling the sample and recording SMF single color data.

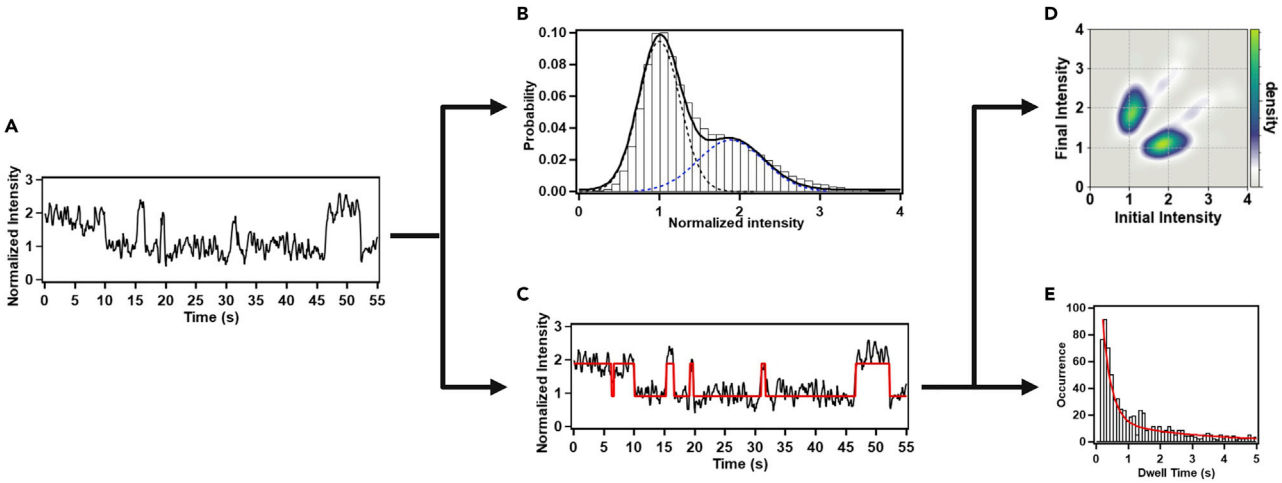

Figure 10. Single-molecule fluorescence data processing workflow

(A) A representative single-molecule fluorescence trajectory of a dynamic apo A_{2A}AR-Cy3 molecule in lipid nanodiscs. Fluorescence intensities fluctuate predominantly between two normalized intensity states of 1 and ~2. The intensity is normalized to the mean intensity of the low-intensity state.

(B) Histogram compiled from nearly 200 dynamic time trajectories of apo A_{2A}AR-Cy3 molecules. Peaks representing individual A_{2A}AR-Cy3 populations are from fitting histograms with Gaussian distributions.

(C) Hidden Markov model fit (red) of the fluorescence intensity trajectory (black) shown in (A) using the 'HaMMy' program.

(D) Transition density plot (TDP) generated from data fit in 'HaMMy'.

(E) Representative dwell-time histogram of measured kinetic rates generated from the HaMMy output. The histograms are fit with an exponential function to generate the red curve. Igor Pro was used to generate and fit the dwell-time histogram.

41. Mount the sample: Place the sample slide on the TIRF stage based attached to inverted IX73 microscope equipped with 60×, 1.20 NA water-immersion objective (Figure 9B).
42. Excite the sample: Use 532 nM laser to excite the sample via a prism attached to the surface of a slide (Figure 9B).
43. Record data: Record emission from Cy3-labeled A_{2A}AR nanodisc complexes on an EMCCD camera with 100 milliseconds integration time. We typically record movies for 100 s to ensure that most molecules are eventually photobleached (Figure 9C), which confirms that the observed particle is a single molecule.
44. For each sample slide, record at least 15–20 movies.
45. Process the data and extract intensity time traces: We use the smFRET package and program "Single", made available from the lab of Prof. Taekjip Ha, for data acquisition and processing. A link to the software is provided below. Additional instructions for using the software are provided in the download.

Single-molecule data analysis

Timing: a week to few weeks depending on the complexity and quality of the experimental data

This section describes the process for quantitatively analyzing the SMF data and generating corresponding histograms and transition density plots used to visualize the data (Figure 10).

46. Select dynamic molecules: Molecules that exhibit single-step photobleaching are selected using the smFRET data analysis package (link below) modified to analyze single-color data written using MATLAB (provided by the laboratory of Taekjip Ha, also see key resources table). Correct the background of each trajectory by the intensity after photobleaching and normalize the fluorescence intensities to the mean intensity of the lowest intensity state. Truncate individual

dynamic trajectories before photobleaching. For each condition (ligand or no ligand added), we typically select approximate 200 molecules (Figure 10A).

47. Create histograms: Generate binned intensity histograms and fit the histograms into Gaussian distributions. We use Igor Pro to generate and fit histograms with Gaussian distributions (Figure 10B).
48. Calculate error: record data from at least five to seven independent movies to calculate the error bars on the fluorescence intensity histograms.
49. Fit the data: Use Hidden Markov model to fit individual normalized trajectories into different conformational states and determine the dwell times spent in each state (Figure 10C). The analysis closely follows published protocols from the Ha lab (McKinney et al., 2006).
50. Generate the transition density plots (TDP) to reveal the probabilities of transition events (Figure 10D).
51. Construct the dwell times histograms and fit with exponential decay function to determine rate constants of transitions (Figure 10E). We use the freely available program HaMMy program (link below) to process single-color data to fit trajectories and generate TDP plots.

EXPECTED OUTCOMES

Expected outcomes are described above and illustrated in Figures 6, 7, and 10. Typical yields of A_{2A}AR are ~3 mg of purified protein per liter of initial cell culture. This yield corresponds to the point in the protocol after purification in detergent and before nanodisc assembly. Typical yields of assembled nanodiscs containing folded and functional A_{2A}AR are 1.5–2 mg from 1 L of initial cell culture.

LIMITATIONS

The protocols described here have been optimized for producing nanodisc samples with human A_{2A}AR and the MSP variant MSP1D1. While the general steps of the described protocols are applicable to additional GPCRs or to preparing nanodisc samples with other membrane scaffold protein variants, details of the process will likely need to be optimized further for other GPCRs. Further, while we have been able to successfully apply the above protocol to producing nanodisc samples with a range of different phospholipids, some membrane components such as sterols are less soluble in detergent buffers used to assemble nanodiscs.

TROUBLESHOOTING

Problem 1

Low yield of expressed MSP1D1 (see "expression and purification of the membrane scaffold protein MSP1D1 for nanodisc production", starting at step 42).

Potential solutions

Be sure not to exceed 4 h induction time after adding IPTG, as extending post-induction time past this point results in significant less expressed MSP. If low expression is still observed, lower the incubator temperature to 30°C after inducing MSP expression.

Problem 2

Low yield of expressed A_{2A}AR (see "large scale expression of A_{2A}AR in *P. pastoris* starting at step 25).

Potential solutions

Extend the post-induction duration of expression and lower the temperature. Post-induction duration of 72 h and temperature at 20°C may result in better yield. It is important to optimize the addition of methanol according to the changed conditions.

Problem 3

Yield of purified A_{2A}AR is low (see "purification of A_{2A}AR for nanodisc assembly", starting at step 32.

Potential solutions

Shorten the extraction period (step 36), i.e., decrease the time the resuspended membranes are mixed with buffer containing detergent to extract the protein. Shortening this period can sometimes improve the yield. Be sure to carry out all protein extraction and purification steps at 4°C or on ice. Take care not to apply more than 400 × *g* force during any protein concentration step and make sure the sample is well mixed between each centrifugation run.

Problem 4

Yield of assembled nanodiscs is low (see "nanodisc assembly and Cy3 labeling for single-molecule fluorescence", starting at step 4).

Potential solutions

There can be more than one reasons for this problem. One of the key factors is MSP:Lipid and Membrane protein:MSP:Lipids ratios. Optimize these ratios by screening various ratios using size exclusion chromatography.

A second potential reason can be incomplete removal of detergent. The presence of detergent can be determined by NMR. If detergent is observed in the sample, increase the duration that Biobeads are incubated with the assembled nanodisc components. Though Biobeads can be reused, applying fresh biobeads may also help.

Also be sure to use a temperature near to the phase transition temperature of the lipid species present in the largest amount for nanodisc assembly.

Problem 5

Non-specific Cy3 labeling of $A_{2A}AR$ (see "nanodisc assembly and Cy3 labeling for single-molecule fluorescence", starting at step 10).

Potential solutions

Incorporation of non-specific fluorescence labels can result in loss of observed transitions in fluorescence intensities and other problems. Avoid incubating the sample with Cy3 for more than 3 h. If non-specific labeling is still suspected, reduce the incubation time to 1–1.5 h or reduce the amount of dye.

Problem 6

Non-specific adsorption of molecules on the slide surface (see "$A_{2A}AR$-nanodisc complex immobilization and addition of ligands", starting at step 35).

Potential solutions

There can be two reasons for this problem. One is from protein aggregates forming on the slide surface because the PEG passivation is not working properly. If this is observed, be sure to use fresh reagents and buffers for PEGylation of the quartz slides.

A second reason can be from using a sample concentration in the channel that is too high. As an example, we typically start with a concentration of $A_{2A}AR$ in solution at 1–2 mg/mL and dilute this sample by approximately a factor of 10,000 to achieve optimal results. Also be sure to add the correct amount of biotinylated PEG, streptavidin, and labeled sample to the slide.

Problem 7

Sample bleaches too quickly (See "single-molecule data acquisition", starting at step 41).

Potential solutions

This is observed if the oxygen scavenging system is not functioning properly. To avoid this, use freshly prepared glucose oxidase and catalase solution.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Matthew Eddy (matthew.eddy@chem.ufl.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The modified single-color data analysis package is available upon request.

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AUTHOR CONTRIBUTIONS

M.T.E. wrote the manuscript with N.T., A.P.R., S.W., and R.L. All authors contributed to the manuscript and approved it for publication.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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