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Reconstitution of Membrane-associated Components of a G-protein Signaling Pathway on Membrane-coated Nanoparticles (Lipobeads)

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[Abstract] G-protein coupled signaling pathways are organized into multi-protein complexes called signalosomes that are located within and on cellular membranes. We describe the use of silica nanoparticles coated with a unilamellar phospholipid bilayer (lipobeads) to reconstitute the activated photoreceptor G-protein α-subunit (Gtα*) with its cognate effector (phosphodiesterase-6; PDE6) for biochemical and structural studies of the activation mechanism regulating this GPCR signaling pathway. Lipobeads are prepared by resuspending dried-down phospholipid mixtures with monodisperse 70 nm silica particles, followed by extrusion through a 100 nm membrane filter. This uniform and supported liposomal preparation is easily sedimented, permitting the separation of soluble from membrane-associated proteins. Upon loading lipobeads with Gtα* and PDE6, we find that activation of PDE6 catalysis by Gtα* occurs much more efficiently than in the absence of membranes. Chemical cross-linking of membrane-confined proteins allows detection of changes in protein-protein interactions, resulting from G-protein activation of PDE6. The advantages of using lipobeads over partially purified membrane preparations or traditional liposomal preparations are generally applicable to the study of other membrane-confined signal transduction pathways.

Keywords: Signalosome, Signal transduction, G-protein, Peripheral membrane protein, Liposome, Protein-protein interactions, Nanoparticle

[Background] Supported phospholipid bilayers have been used for a variety of fundamental and applied applications, including membrane biophysics, biosensors, cell-cell interactions, screening assays of membrane proteins, and as drug-delivery vehicles (Ng *et al*., 2004; Troutier and Ladaviere, 2007; Chemburu *et al*., 2010; Crites *et al*., 2015; Schadauer *et al*., 2015). Microspheres consisting of silica or polymeric materials (*e.g*., hydrogels) have been used as a solid support for forming unilamellar phospholipid bilayers whose size, density, membrane curvature, and other biophysical properties can be optimized for their intended applications.

We have utilized 70 nm diameter silica particles coated with a unilamellar lipid bilayer (termed “lipobeads”; Figure 1A) to bind the prenylated photoreceptor phosphodiesterase (PDE6) holoenzyme (Figure 1B), as well as the acylated photoreceptor G-protein α-subunit (Gtα), for enzymological and structural studies of the Gtα*-PDE6 activation complex in a membrane-confined environment (Figure 1C) (Irwin *et al*., 2019). Activation of PDE6 by its cognate G-protein (transducin) occurs on the membrane surface in the light-harvesting outer segment of rod and cone photoreceptors upon

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photoactivation of rhodopsin (Cote *et al*., 2021). Studies have shown that the efficiency with which Gtα* can activate PDE6 is greatly enhanced when both proteins are tethered to either the photoreceptor membrane or to liposomal preparations (Melia *et al*., 2000). Reduction in the dimensionality of diffusional encounters between Gtα* and PDE6, in conjunction with high local protein concentrations, likely account for this phenomenon. The ability to reconstitute the proteins comprising GPCR signalosomes *in vitro* on well-defined lipobeads enables the integration of biochemical, biophysical, and structural studies to advance our mechanistic understanding of GPCR signaling pathways, in the context of their membrane environment.

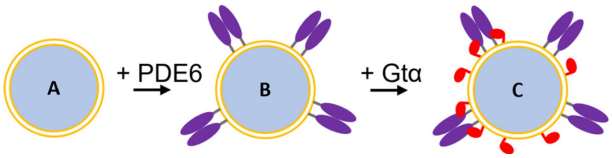

Figure 1. Reconstitution of the Gtα*-PDE6 activation complex on lipobeads.

A. Lipobead composed of a silica nanoparticle coated with a unilamellar phospholipid bilayer. B. PDE6 (purple) attached to lipobeads by their prenyl moieties. C. Addition of acylated Gtα* (red) to lipobeads with pre-bound PDE6 results in Gtα* binding and the formation of the Gtα*-PDE6 activation complex.

Materials and Reagents

1. Amicon Ultra-4 30 kDa or 50 kDa ultrafiltration units (Millipore-Sigma, catalog numbers: UFC803024 and UFC805024)
2. Phospholipids (Avanti Polar Lipids, Inc.)
   a. DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt; Avanti Polar Lipids, catalog number: 890890C-200 mg) is supplied as 100 mg portions dissolved in chloroform at 25 mg/mL (FW 698.5; 35.8 mM).
   b. DOPC: 1,2-dioleoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, catalog number: 850375C-500 mg) is supplied as 100 mg portions dissolved in chloroform at 25 mg/mL (FW 786.1; 31.8 mM).
3. Monodisperse silica particles [70 nm diameter; 25 mg/mL aqueous suspension (0.11 μM; 7×10^{13} particles/mL); density = 2.0 g/cm^3] are obtained from DiagNano (CD Bioparticles, catalog number: DNG-B004).
4. Purified PDE6 is prepared as described previously (Pentia *et al*., 2005) and stored at -20°C in PDE6 storage buffer.
5. Purified Gtα-GDP or purified Gtα*-GTPγS is prepared as described previously (Ting *et al*., 1993; Irwin *et al*., 2019) and stored at -20°C in Gtα storage buffer.
6. NuPAGE 4-12% Bis-Tris gel (Invitrogen, catalog number: NP0321BOX)
7. Bis(sulfosuccinimidyl) suberate (BS3) (Thermo Scientific, catalog number: 21580)
8. NaCl (Electron Microscopy Sciences, catalog number: EM-7760)

9. $MgCl_2 \cdot 6H_2O$ (Electron Microscopy Sciences, catalog number: EM-5980)
10. Dithiothreitol (Electron Microscopy Sciences, catalog number: EM-3860)
11. Glycerol (IBI Scientific, catalog number: IB15760)
12. Tris base (J.T. Baker, catalog number: JT4109)
13. $AlCl_3 \cdot 6H_2O$ (Millipore Sigma, catalog number: A0718)
14. NaF (J.T. Baker, catalog number: JT3689)
15. HEPES, hemisodium salt (MP Biomedicals, catalog number: IC15245180)
16. Guanosine-5'-diphosphate, sodium salt (Millipore Sigma, catalog number: G7127)
17. HNM buffer (see Recipes)
18. PDE6 storage buffer (see Recipes)
19. Gtα storage buffer (see Recipes)
20. 745 μM $AlCl_3$ in HNM buffer (see Recipes)
21. 250 mM NaF in HNM buffer (see Recipes)
22. 1 M $MgCl_2$ (see Recipes)
23. 50 mM GDP (see Recipes)
24. 10 mM BS3 (see Recipes)
25. 0.5 mM BS3 (see Recipes)

Equipment

1. Eppendorf 5415D centrifuge
2. Vortex mixer (VWR, catalog number: 10153-838)
3. Avanti Mini Extruder Set (Avanti, catalog number: 610023)
   a. 0.1 μm, 19 mm diameter polycarbonate membrane (Avanti, catalog number: 610005 or Whatman catalog number: 800309)
   b. 10 mm filter supports (Avanti, catalog number: 610014)
   c. 1000 μL gas-tight syringe (Avanti, catalog number: 610017)

Procedure

A. Preparation of phospholipids

1. An 80:20 molar ratio of DOPC:DOTAP (Melia *et al.*, 2000) is prepared by combining 1.26 mL of DOPC (31.5 mg, 40 μmol) and 279 μL of DOTAP (7.0 mg, 10 μmol) with 461 μL of chloroform. [DOPC] = 20.0 mM, [DOTAP] = 5.0 mM; total [phospholipid] = 25.0 mM
2. Aliquot 200 μL portions (4.0 μmol DOPC and 1 μmol DOTAP) into glass tubes and evaporate them under a stream of nitrogen gas.
   Dried down lipids are stored in darkness at -20°C for up to six months until use.

B. Preparation of silica nanoparticles

1. Silica particles are disaggregated by mixing 200 µL (5 mg; 1.4×10^{13} particles) of silica particles with 1 mL of 95% ethanol.
   a. Vortex at maximum speed for 2 min.
   b. Incubate for a minimum of 1 h at room temperature (overnight is preferable).
2. Centrifuge the silica suspension at 10,000 × *g* for 10 min in the Eppendorf 5415D centrifuge at 10°C.
3. Resuspend the pellet with 1.0 mL of ice-cold HNM buffer, vortex for 2 min, and centrifuge again at 10,000 × *g* for 10 min in the Eppendorf 5415D centrifuge at 10°C, to remove residual ethanol.
4. Resuspend the pellet with 1.0 mL of ice-cold HNM buffer, vortex for 2 min, and keep on ice until use.

C. Preparation of lipobeads
1. The dried-down DOPC/DOTAP lipid mixture is mixed with the silica particle suspension at room temperature without nitrogen gas, and gently vortexed (setting #5-6) for 2 min, then allowed to settle for 1 min. This process is repeated four more times.
   Final concentration: 80:20 DOPC:DOTAP = 5 mM (total phospholipid), [lipobead] = 23 nM
2. Assemble the Mini Extruder (a video created by the manufacturer describing the assembly of the Mini Extruder is found here: https://www.youtube.com/watch?v=8ei62ZmLgSM):
   a. Pre-wet two 10 mm filter supports with HNM buffer and place one inside the O-ring of the internal membrane support.
   b. Place the membrane support into the outer casing.
   c. Place a single polycarbonate 0.1 µm membrane on top of the membrane support.
   d. Place the second filter support on the second membrane support and place the second membrane support into the outer casing (O-rings facing each other).
   e. Place the Teflon bearing into the retainer nut.
   f. Finger-tighten the retainer nut onto the outer casing.
   g. Attach an empty gas-tight syringe to one end of the Mini Extruder.
   h. Pre-wet the assembly by passing HNM buffer through the Mini-Extruder with the second gas-tight syringe.
3. Extrude the lipid/silica suspension through the Mini Extruder:
   a. Load the lipid/silica mixture into one of the gas-tight syringes.
   b. Firmly depress the syringe barrel to force the lipobeads through the membrane quickly (~10 s duration per cycle)
   c. Repeat a total of 15 times, with the lipobead suspension finally filling the originally empty syringe (this prevents aggregates that never passed through the filter from being recovered).
4. Separate the unilamellar lipobeads recovered from the syringe from unbound phospholipids by centrifugation at 10,000 × *g* for 10 min at room temperature in the Eppendorf 5415 D centrifuge.
5. Resuspend the pellet with 500 µL of HNM buffer for a lipobead concentration of 46 nM.
6. Lipobead preparations are stable for ~2 days if stored in the dark at room temperature.

D. Binding PDE6 to lipobeads

1. Add 10 µL of 46 nM lipobead preparation (0.46 pmol) to purified PDE6 holoenzyme and incubate at room temperature for 30 min.
   a. The amount of PDE6 added to the lipobead preparation can be varied from 10 fmol (1 nM concentration) to at least 10 pmol (1 µM concentration), without exceeding the maximum binding capacity of the lipobeads. The amount of PDE6 added to the lipobeads is determined as described in Step G2b.
   b. The glycerol concentration of the PDE6 stock solution must be less than 10% (v/v) for optimal binding to lipobeads.
   c. To reduce the glycerol concentration, the stock solution of PDE6 can be buffer exchanged using 50 kDa MWCO Amicon Ultra-4 filtration units. The protein is diluted over ten-fold with HNM buffer, followed by centrifugation at 3,000 × *g* for 10-20 min. Centrifugation time will depend on the initial volume of protein.
2. Spin the PDE6-lipobead mixture at 10,000 × *g* for 10 min in the Eppendorf centrifuge in the cold room (~10°C).
   a. Remove supernatant as soon as possible after centrifugation, as the pellet loosens over time.
   b. To determine the extent of PDE6 binding to lipobeads, the supernatant can be recovered and assayed. As seen in Figure 2, over a range from 0.01 to 10 pmol of PDE6 added, 85 ± 6% is bound to lipobeads.

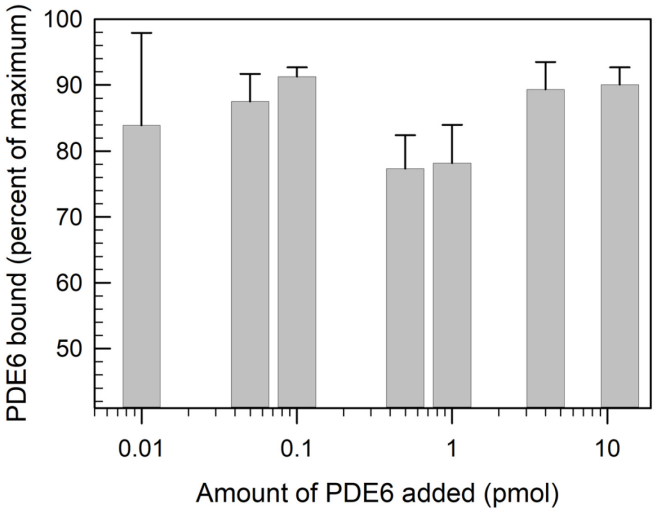

Figure 2. Binding of PDE6 to lipobeads.

Lipobeads (0.46 pmol) are mixed with 40 µL of the indicated amount of PDE6 and incubated for 30 min. Following centrifugation, the supernatant fractions are assayed for PDE6 activity (see Step G2b), to calculate the percent of total PDE6 activity bound to the lipobeads. Data represent the mean ± S.D. (n = 3).

3. Use the lipobead pellet containing bound PDE6 for the subsequent incubation with Gtα*.

E. Preparation of aluminum fluoride-activated Gtα* (Gtα-GDP-AlF_4^-)
   1. For Gα-GDP preparations stored in 50% (v/v) glycerol, the glycerol concentration is first reduced by buffer exchange using HNM buffer and ultrafiltration.
      a. The glycerol concentration of the Gtα preparation must be less than 10% (v/v) for optimal binding to lipobeads.
      b. Perform buffer exchange by first diluting the Gtα stock solution over ten-fold with HNM buffer, placing the protein sample in the upper chamber of a 30 kDa MWCO Amicon Ultra-4 filtration unit, and centrifuging at 3,000 × *g* for 10-20 min (depending on the initial volume of the diluted protein).
   2. Purified Gtα-GDP is activated by the addition of aluminum fluoride (Bigay *et al.*, 1987). In a final volume of 50 µL, add the following:
      a. 45.6 µL of Gtα-GDP in Gtα storage buffer [containing <10% (v/v) final glycerol concentration].
      b. 2.0 µL of 745 µM $AlCl_3$ (final concentration = 30 µM).
      c. 2.0 µL of 250 mM NaF (final concentration = 10 mM).
      d. 0.4 µL of 1 M $MgCl_2$ (final concentration = 8 mM).
      e. Incubate on ice for 15-30 min.

F. Formation of Gtα*-PDE6 complex on lipobeads
   1. Add 50 µL of Gtα* (either Gtα-GDP-AlF_4^- or Gtα-GTPγS) to the PDE6-containing lipobead pellet, mix well, then incubate for 45 min at room temperature.
      a. The concentration of Gtα* added to the PDE6-bound lipobeads can be varied up to a maximum of ~5 µM (this being the limit of solubility of purified Gtα that is added to the lipobead mixture).
      b. For chemical cross-linking experiments (see Figure 3), the cross-linker is added to the lipobead suspension prior to centrifugation. Add BS3 to a final concentration of 0.125 mM, from a 0.5 mM working solution or 10 mM stock solution. Allow crosslinking reactions to proceed for 45 min at room temperature, followed by centrifugation at 10,000 × *g* for 10 min. Resuspend the pellets in 1× gel loading sample buffer for SDS-PAGE.
      c. For enzymatic assays of cGMP hydrolysis of PDE6 (see Figure 4), the lipobeads containing Gtα*-PDE6 are not centrifuged prior to addition of cGMP substrate to initiate the assay of PDE6 hydrolytic activity.

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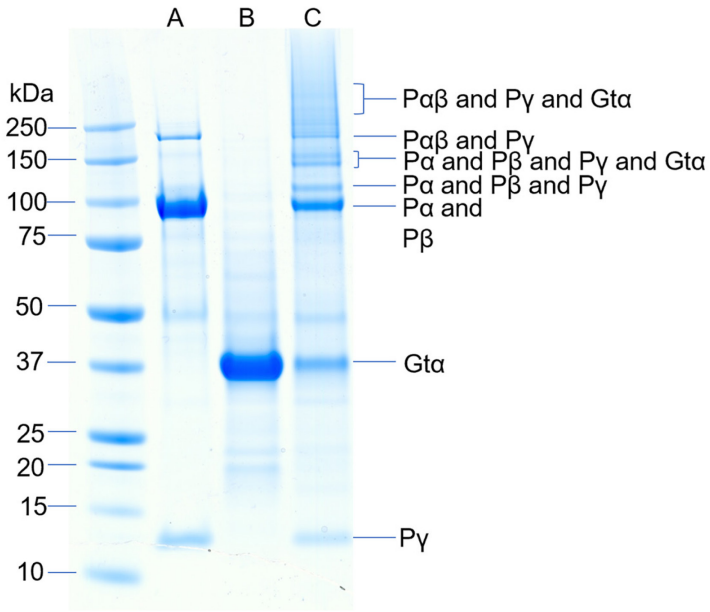

Figure 3. Chemical cross-linking of the activated complex of Gtα and PDE6.

Cross-linking of PDE6 and Gtα* was performed by first binding 9 pmol of PDE6 to 0.46 pmol lipobeads for 20 min followed by centrifugation. The lipid pellet was resuspended with 115 pmol Gtα-GDP-ALF and incubated for 45 min followed by centrifugation. The pellet was resuspended in 20 µL of HNM buffer containing 0.125 mM BS3 and crosslinking proceeded for 45 min. Lane A contains purified PDE6, lane B contains purified Gtα*, and lane C contains the crosslinked mixture.

G. Analytical methods

1. Determination of protein concentration

   The concentration of PDE6 or Gtα is determined by the bicinchoninic assay (Thermo Fisher, Inc.) following the manufacturer's protocol.

   For analysis of Gtα samples containing excess unbound nucleotide, buffer exchange using Amicon Ultra-4 30 kDa ultrafiltration units (following the procedure described in Step E1b) with buffer lacking guanine nucleotides is performed, to remove interference with the protein assay.

2. PDE6 enzymatic activity assay

   a. Quantify the rate of cGMP hydrolysis using a colorimetric assay of inorganic phosphate, in which the breakdown of cGMP to 5'-GMP by PDE6 is followed by addition of 5'-nucleotidase to liberate inorganic phosphate (Cote, 2000).

   b. To measure the total amount of PDE6 present in a sample, incubate the enzyme with trypsin to proteolytically digest the inhibitory γ-subunit that is tightly bound to the holoenzyme (Pentia *et al.*, 2005). Calculate the amount of PDE6 in the sample with knowledge of the catalytic turnover number for trypsin-activated PDE6 [kcat = 5600 cGMP hydrolyzed per s per PDE6 (Mou *et al.*, 1999)].

c. To calculate the percent of maximal activation induced by addition of Gtα (see Figure 4), the extent of transducin activation of PDE6 catalytic activity on lipobeads is referenced to the maximum trypsin-activated enzyme activity (Step G2b).

d. Measure the PDE6 basal (*i.e.*, nonactivated) activity on lipobeads in the absence of added Gtα*.

Data analysis

A. Evaluation of the ability of Gtα* to activate PDE6 catalysis.

1. There is substantial evidence in the literature (Malinski and Wensel, 1992; Melia *et al.*, 2000) supporting the importance of membrane attachment for efficient activation of PDE6 by Gtα*. Membrane binding of the G-protein and its effector reduces the dimensionality of diffusional encounters, and also creates a high local concentration of these interacting proteins.
2. Figure 4 demonstrates that Gtα* activation of PDE6 in solution (blue symbols) is much less effective than when the same concentration of PDE6 and Gtα* are pre-incubated with lipobeads prior to assaying PDE6 activation (black symbols). Figure 4 also shows that the efficacy with which Gtα* can activate PDE6 is greatly enhanced as the PDE6 concentration bound to lipobeads increases (compare black and red symbols). Under these conditions, a ten-fold increase in PDE6 bound to lipobeads reduces by five-fold the concentration of Gtα* needed to achieve 50% activation of PDE6.

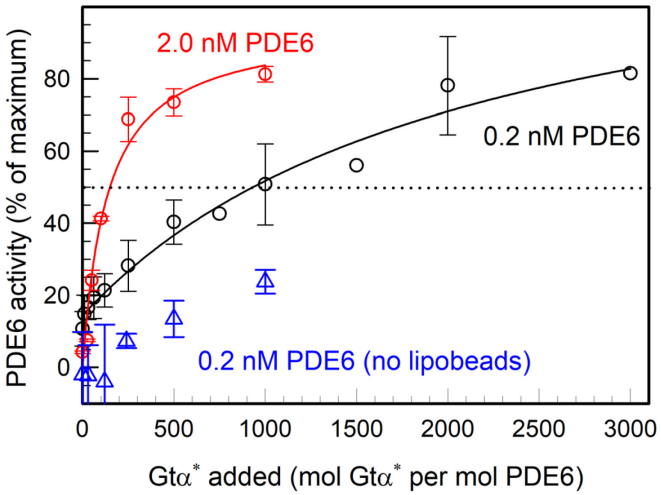

Figure 4. G-protein activation of PDE6 is enhanced on lipobeads in a concentration-dependent manner.

PDE6 holoenzyme (either 0.2 nM or 2 nM) was incubated with 1.1 pmol of lipobeads for 30 min at room temperature, prior to the addition of the indicated amounts of Gtα-GTPγS (Gtα*) for 45 min. A separate sample of PDE6 was mixed with Gtα* in the absence of lipobeads. Upon addition of cGMP (2 mM final concentration), the extent to which PDE6 was activated by Gtα* was determined by monitoring the degradation of cGMP. The extent of activation by Gtα* is

expressed as the percentage of PDE6 activity measured when the inhibitory Pγ subunits of PDE6 are removed by limited trypsin proteolysis. Data represent the mean ± S.D. for 3 experiments (blue and black symbols; three black symbols without error bars are individual determinations) or the mean and range for 2 experiments (red symbols).

B. Characterization of protein complex formation using chemical cross-linking.
   1. Protein complex formation is determined by the appearance of higher molecular weight bands on SDS-PAGE following chemical crosslinking (Figure 2).
   2. The identity of proteins present in various cross-linked complexes (Figure 2) is ascertained by excising Coomassie-stained bands from the gel, performing digestion with trypsin, and analysis by mass spectrometry (see Irwin *et al*., 2019 for details).
   3. Identification of cross-linked peptides enables integrative structural modeling of the Gtα*-PDE6 complex on lipobeads, as described in detail in Irwin *et al*. (2019).

Notes

Lipobead properties:

1. A single 100 nm unilamellar liposome encapsulating a silica particle (surface area = 0.031 μm^2) is calculated to contain 80,047 phospholipids per particle [using Encapsula NanoSciences calculator (https://encapsula.com/calculator)].
   This calculation is based on knowledge of the phospholipid head-group area [for phosphatidylcholine, 0.71 nm^2 (Kucerka *et al*., 2006)] and the thickness of the lipid bilayer [5 nm (Marquardt *et al*., 2016)].
2. Distribution of phospholipids in unilamellar membrane is calculated to be 55% in the outer monolayer and 45% in inner monolayer, in agreement with ^{31}P NMR spectroscopy of 100 nm unilamellar liposomes prepared by extrusion (Malinski and Wensel, 1992).
3. Total phospholipid concentration encapsulating the silica particles: 46 nM particles × 80,047 lipids per particle = 3.7 mM; thus, a 2.7-fold excess of phospholipids are present when initially preparing lipobeads.
4. Surface-accessible (*i.e*., outer monolayer) phospholipid concentration = 2.0 mM.
5. The stability of the lipobead preparations (as judged by the extent of activation of PDE6 by Gtα*, as well as the extent of PDE6 binding to lipobeads) has been found to decline significantly three days after initial preparation and storage of lipobeads at room temperature.

Recipes

1. HNM buffer (1 L)
   20 mM HEPES-0.5Na (4.986 g)
   100 mM NaCl (5.844 g)

2 mM $MgCl_2·6H_2O$ (0.407 g)
pH 7.5

2. PDE6 storage buffer (1 L)
   20 mM HEPES-0.5Na (4.986 g)
   100 mM NaCl (5.844 g)
   2 mM $MgCl_2·6H_2O$ (0.407 g)
   2 mM DTT (0.308 g)
   50% (v/v) glycerol
3. Gtα storage buffer (1 L)
   10 mM Tris base (1.211 g), pH 7.4
   2 mM $MgCl_2·6H_2O$ (0.407 g)
   2 mM DTT (0.308 g)
   Add 50 µM GDP [1/1,000 dilution of 50 mM GDP and 40% glycerol (v/v)] immediately prior to storage at -20°C.
4. 745 µM $AlCl_3$ in HNM buffer
   $AlCl_3$ (9.9 mg) in 100 mL of HNM buffer
5. 250 mM NaF in HNM buffer
   NaF (105 mg) in 10 mL of HNM buffer
6. 1 M $MgCl_2$
   $MgCl_2·6H_2O$ (2.033 g) in 10 mL of water
7. 50 mM GDP (1 mL)
   GDP-Na (23.3 mg) in 10 mM Tris, pH 7.5
8. 10 mM BS3 (175 µL)
   1 mg BS3 in 175 µL of water
9. 0.5 mM BS3 (20 µL)
   1 µL of 10 mM BS3 diluted to 20 µL with HNM buffer

Acknowledgments

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Competing interests

The authors declare no competing financial or non-financial interests.

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