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WORKS FOR ME 1

Golgi immunopurification (Golgi-IP) for subcellular lipid profiling

COMMENTS 0

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ABSTRACT

The Golgi is a membrane-bound organelle that is central to protein and lipid processing, sorting and secretion in the cell. Despite its critical cellular function, there has been challenges to quantitatively assess Golgi lipid profiles. To overcome this hurdle, we developed a rapid harvesting and purification method using immunoprecipitation (Golgi-IP). This protocol provides details for preparing Golgi-IP lipidomics samples.

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MATERIALS TEXT

Consumables

- Marker pen
- Pipette set (1000 µl)
- PPE kit (Lab coat, gloves, safety glasses)
- Ice and ice bucket
- 1.5 ml Eppendorf tubes rack

Reagents

- Anti-HA magnetic beads (Thermo Fisher Scientific, cat. no. 88837)
- KPBS (136 mM KCl, 10 mM KH2PO4, pH 7.25 using KOH in Optima LC/MS water)
- Splashmix (SPLASH® LIPIDOMIX® Mass Spec Standard, cat. no. 330707)
- 0.9% (w/v) saline (VWR, cat. no. S5825)

Equipment

- Glass Vessel: (VWR, cat no. 89026-386)
- Tissue Grinder: (VWR, cat no. 89026-398)
- Benchtop centrifuge (VWR)
- Milli-Q water system
- ID-X Orbitrap Tribrid Mass Spectrometer

SAFETY WARNINGS

Please refer to Safety Data Sheets (SDS) for health and environmental hazards.

Preparation of homogenizers and sample tubes

1 Wash the glass vessel homogenizer with MilliQ Water, 10 times each. Wash the tissue grinder homogenizer thoroughly with DI Water and MilliQ Water, especially the gap between the white parts, don't touch the part that goes into the glass vessel. Then dry upside-down using paper towels. Carefully place the glass vessels against something to prevent falling down. Minimize any contact between the grinder and anything else.

2 Prepare microcentrifuge tubes as follows on a metal rack on ice (for each sample, from left to right): ① 2 mL tube for cell suspension from harvesting; ② 1.5 mL tube for post-homogenization cell suspension (organelles in supernatant, membranes in pellet); ③ 1.5 mL tube for whole cell sample; ④ 1.5 mL tube for beads; ⑤ 1.5 mL tube for post-magnetic samples; ⑥ 1.5 mL tube for final Golgi-IP samples. Carefully label tubes ③ and ⑥ with detailed samples and experiments names.

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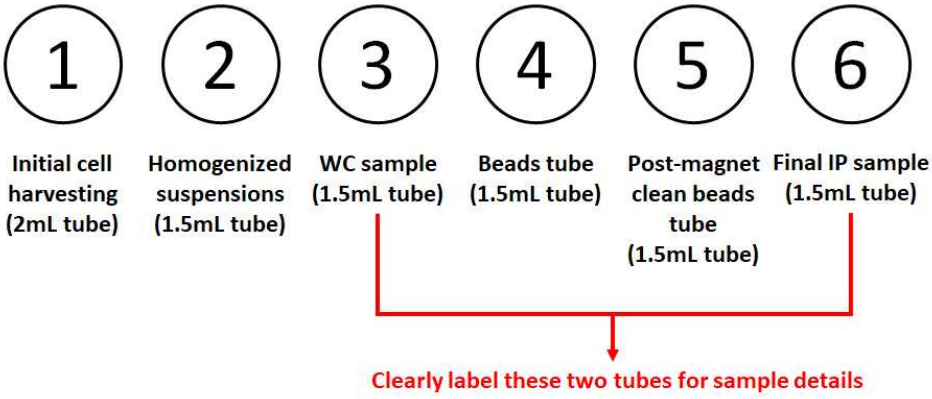

WC: whole cell; IP: immunoprecipitate

Preparation of Anti-HA beads

3 Pool all required volumes together (100 µL / plate, e.g. 800 uL total for 8 plates, extra is not needed).

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4 Shake bottle very well before removing as beads tend to sink to the bottom.

5 Wash 3 x with the same volume cold clean MS grade KPBS, after settling on magnet. Remove the holder from the magnet itself before dispending washing KPBS to avoid wetting the magnet.

6 Resuspend with KPBS with same amount of volume originally removed from bottle.

7 Aliquot 100 µL into each 1.5 mL labeled tubes ④.

Cell preparation before harvesting

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8 Wash the first set of 15cm plates (each set has two plates) with 10 mL of DMEM/plate (for HEKS, use no serum + no antibiotics).

9 Replace with 10 mL of DMEM/plate for an hour. You can also use full media or other treatments based on your experimental needs.

20m

10 10 mL The second set of plates will be washed 00:20:00 later after the first set and so on.

Note

Note: it is very important to maintain the schedule of the plate washing --> Golgi-IP. If there are any deviations to the schedule for any reason, note them as it may affect results of experiment.

11 One hour after DMEM wash, take the first set of plates from incubator to bench and place on ice.

12 Decant the media. Then Wash the cells twice by pouring ~ 5 mL cold clean MS grade PBS on the edge of the plate, decant the first time and then aspirate the second time.

Cell harvesting

13 Add 950 µL of cold KPBS to each 15-cm dish.

14 Scrape the cells down to the bottom of your plates with a cell lifter and transfer the cell suspension into the 2ml tube ①. Note: this step should be carefully accounted for and done the same between plates. Visually check (with an angle) that all cells have been harvested. We are using a 2mL tube since 950 uL KPBS + cells gives around 2mL volume.

2m

15 Spin at 1000 x g for 00:02:00 at 4 °C.

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Note

Note: centrifuge must already be cooled to 4 °C at this point.

16 Aspirate the supernatant and resuspend the pellets with 950 µL cold KPBS.

17 From this resuspended sample, take 25 µL for whole cell in the 1.5 mL tube ③.
Note: if pellet mixer is used instead of douncer, resuspend the pellets with 100 µL cold KPBS in step 16, homogenize cells and then replenish to 950 uL and follow step 17.

Homogenization and Golgi-IP

18 Transfer the remainder (925 µL) of cells into a clean and pre-chill douncer. Dounce the cells 25 times (for HEK293T cells, other cells need to be optimized) gently on ice and avoid making bubbles.

Note

Note: count and repeat for each of the samples (both in number and speed).

19 Use 2 mL serological pipet to transfer sample from douncer into the 1.5 mL tubes ②.

2m

20 Spin 1,000g for 00:02:00 at 4 °C.

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21 a. Wash douncers during this spin for subsequent harvesting

22 Put the remaining supernatant (**it contains the organelles**) on the 1.5 ml tube ④ with beads and resuspend by pipetting up and down ONE TIME.

Note

Note: leave the pellet and make sure not to accidently suck up any of the pellet as that can negatively affect experiment.

23 Rock in cold room for 00:03:00 (everything from now on is in the cold room). 3m

Note

Note: Max speed. Set timer for 3 mins.

24 Put the ④ tube on magnet. Count at least 00:00:25 to allow for beads to be pulled by magnets. 25s

Note

Note: it is important to keep this count the same between each wash and each sample for consistency I.e. 25 seconds each time .

25 Wash the bound fraction 3 times with 1 mL cold KPBS. Then aspirate all cold KPBS.

Note

Note: during the first wash, make sure to aspirate any liquid trapped on the inner side of the cap. Pipet up and down 2 or 3 times and keep consistent each wash, each sample. After the second wash, resuspend and then switch to the clean 1.5 mL tube ⑤ for the third wash (this step helps reduce background signal) .

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10m

Processing of nonpolar lipids samples

10m

26 For nonpolar metabolites (lipidomics), both IP and WC samples, resuspend in 1000 µL of chloroform:methanol at ratio of 2:1 (v/v) with 1000x diluted Splashmix (Avanti). Then incubate for 00:10:00 .

10m

27 After 00:10:00 of finishing the last IP, place IP samples in the tube ⑤ on the magnet, collect supernatant, and transfer to the 1.5 mL tube ⑥.

1h 40m

28 For both WC and Golgi-IP samples, vortex for 01:00:00 in cold room. Then add 200 µL of 0.9% (w/v) saline (VWR) and vortex for another 00:10:00 in cold room. The mixture was centrifuged at 3000 x g for 00:05:00 at 4 °C . Then discard the top layer (MeOH and saline polar phase) and use bubbeling method to retrieve 600 µL from the bottom layer (chloroform containing lipids) to clean prechilled eppendorf tubes. Next speedvac the chloroform samples until dried. Then store lipidomics WC and IP samples at -80 °C . On the day of analysis, dried lipid extracts were reconstituted in 50 µL of ACN:IPA:water 13:6:1 (v/v/v) and vortexed for 00:10:00 at 4 °C . Then samples are centrifuged for 00:15:00 at 4 °C at max speed, and then 45 µL of supernatant is transferred into glass insert vials for LC/MS.

Note

Note: preparing lipidomics samples takes longer time. Plan accordingly. Can stop after step 28 or speedvac drying step, then store samples at -80C if needed.

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