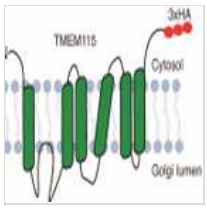

Version 1

Jul 13, 2022

Introducing GolgiTAG to Cells and Immunoprecipitation of Golgi V.1

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ABSTRACT

Golgi apparatus is essential to the secretory pathway of the cell and by extension helps to maintain cellular homeostasis. It is the main transportation hub of the cell, where molecules destined for other organelles within the cytoplasm or outside the cell for secretion are packaged into vesicles. The purification of Golgi using available techniques is time-consuming and laborious, as it requires various centrifugation steps, sometimes in gradient buffers, which reduce yield, compromise its integrity, and increase the chance of contamination, especially from endoplasmic reticulum. Furthermore, depending on the cell type or tissue, the methods for Golgi purification differs. Here, we present a protocol for rapidly purifying Golgi from the mammalian HEK293 cell line, using high affinity anti-HA magnetic beads. This method relies on the immunoprecipitation, in phosphate buffer, of HA-tagged integral membrane protein of the Golgi complex, TMEM115. The HA-tagged TMEM115 expressing vector is packaged into a lentivirus, therefore various mammalian cell lines can be transduced, giving stable expression levels. Our protocol is fast, approximately 10 min, and can be used on various cell lines and tissues without any modification. The Golgi purified using this method are highly enriched, intact, contaminant-free and, depending on solubilisation buffer, could be used for various downstream applications, such as proteomics and metabolomics.

ATTACHMENTS

Golgi stack showing TMEM115 Monther.tif

DOI

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PROTOCOL CITATION

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KEYWORDS

golgi, golgi apparatus, anti-HA magnetic beads, immunoprecipitation, IP, TMEM115, ASAPCRN

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Fig. 1

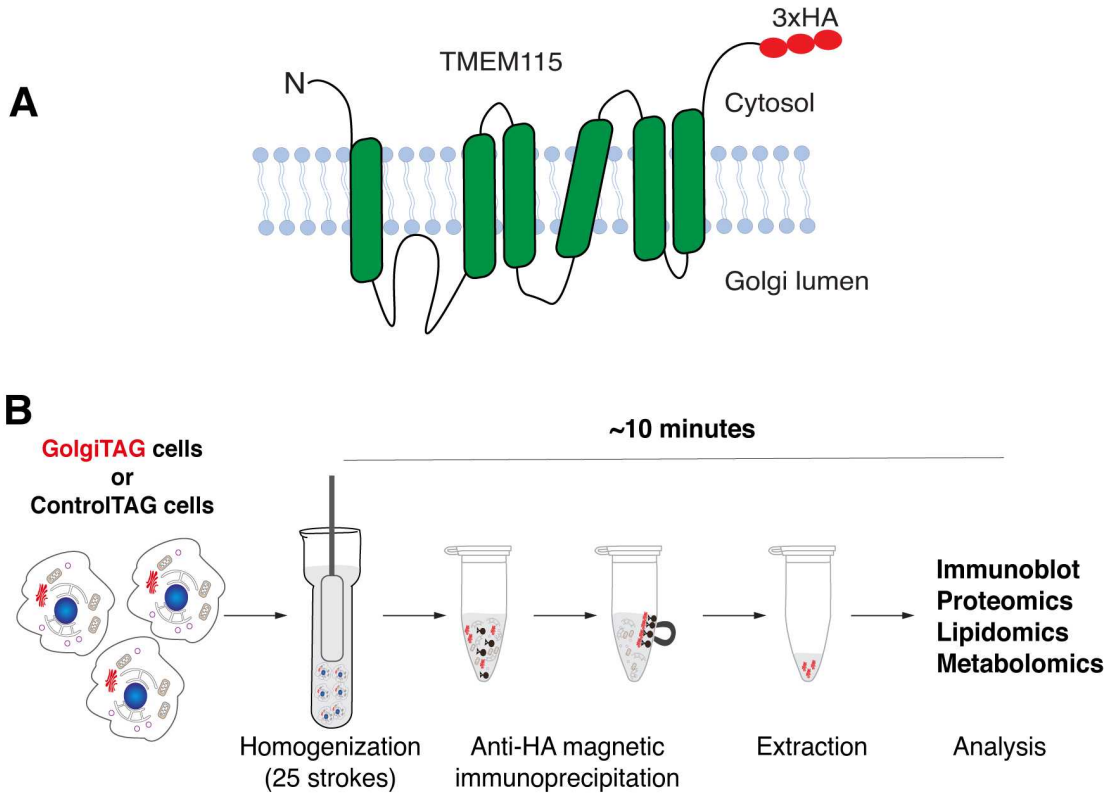

Figure 1:GolgiTag immunoprecipitation.
(a)The sketch diagram of a Golgi cisternae showing the integral protein TMEM115 (green) with 3xHA tag (red) at the C-terminus. Both the N- and the C-terminal are cytosolic. (Note: Diagram is not to scale).
(b)The flowchart of the Golgi Immunoprecipitation. Cells are lysed in KPBS using Dounce homogeniser, before Golgi is captured using magnetic anti-HA beads. (Note: Diagrams are not to scale)

MATERIALS TEXT

Materials

1. Cell lines

1. HEK293FT for virus packaging and propagation (Invitrogen™. Catalog# R70007)
2. HEK293 (ATCC Catalog# CRL-1573)

2. Plasmids

1. pLJC5-KOZAK-TMEM115-3HA (DU68534 available at MRCPPU depository at MRCPPUreagents@dundee.ac.uk). This is the GolgiTag expression construct
2. pLJC5-KOZAK-3HA-Empty (DU70022 available at MRCPPU depository at MRCPPUreagents@dundee.ac.uk). This is the ControlTag expression construct
3. pVSVG. Lentivirus envelope plasmid. Lenti-X HTX Packaging system (Clonetech. Catalog# 631247).
4. pGag/Pol. Lentivirus Gag/Pol plasmid. Lenti-X HTX Packaging system (Clonetech. Catalog# 631247).

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3. Media and Reagents

1. Growth Media: Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO 11960-085); 10% Foetal Bovine Serum (FBS) (Sigma F7524 Batch# BCBW6817); 1% L-Glutamine (GIBCO 25030024); 1% PenicillinStreptomycin (GIBCO 15140122).
2. Selection Media: Growth Media with 2ug/ml Puromycin (Sigma P9620).
3. Transfection media: OptiMem (GIBCO 31985-062)
4. Dulbecco's phosphate-buffered saline (PBS) (GIBCO 14190169)
5. KPBS Buffer: 136mM KCL, 10 mM KH2PO4. Adjust to pH 7.25 with KOH. (Note On the day of use, add Roche cOmplete protease inhibitor cocktail tablet (REF# 11873580001) and Roche PhosSTOP tablet (REF# 04906837001)
6. Lysis Buffer: 50mM HEPEs-KOH pH 7.4; 40mM NaCl; 2mM EDTA; 1.5mM NaVO4; 50mM NaF; 10mM NaPyrophosphate; 10mM NaBetaGlycerophosphate; 1% TritonX100. (Note On the day of use, add Roche cOmplete protease inhibitor cocktail tablet (REF# 11873580001)
7. Pierce™ BCA Protein Assay Kit (Ref# 23227. Lot# VA294738)
8. Linear polyethylenimine (PEI Max 40K. Polyscience #24765)
9. Polybrene Infection/Transfection reagent (Millipore TR1003G)
10. NuPAGE 4xLDS sample buffer (Invitrogen. Lot #1941674)
11. 2-Mercaptoethanol (Sigma-Aldrich M6250)

4. Equipment

1. Homogenizer, 2-ml vessel (VWR, Catlog# 89026-386) and plain plunger (VWR, Catlog#. 89026-398)
2. Orbiter (The Belly Dancer Shaker; IBI Scientific, model # BDRAA115S)
3. DynaMag™ Spin Magnet. (Thermofisher scientific. Catalog #12320D)
4. Incubator with FPI-sensor system and display controller MB1 (BINDER GmbH. Model: CB150. Power Output: 1.40kW, 230V, 6.1 Amp). This incubator has CO_2 and O_2 control.
5. Microcentrifuge with thermostat (VWR Micro Star 17R. S/N 42209232. REF# 521-1647)

5. Consumables

1. Pierce™ Anti-HA magnetic beads (Thermofisher. Catalog # 88837)
2. 10cm and 15cm tissue culture Petri Dishes(ThermoFisher. Catalog# 172931 and 168381 respectively).
3. 1.5ml low binding Eppendorf tubes (Sarstedt. REF # 72.706.600).
4. 15ml CELLSTAR® tubes (Greiner bio-one. Catalog# 188271).
5. 50ml CELLSTAR® tubes (Greiner bio-one. Catalog# 227261).
6. Standard 1ml and 200µl Pipette tips (Greiner bio-one. Catalog# 686271 and 685261 respectively).
7. Syringe filter (0.45µm. Sartorius, Item # ST16537-Q)
8. Syringes (10ml) (Medicina. REF# IVS10. LOT# 19111004).
9. Disposable Cell Lifter (FisherBrand. Catalog # 08-100-240)

SAFETY WARNINGS

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

Handle lentivirus under sterile condition in a CATEGORY 2 biological safety cabinet.

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Packaging GolgiTag and ControlTag construct into lentivirus system 2d 0h 35m

1

This is done under sterile condition in a CATEGORY 2 biological safety cabinet

2 Grow 2 dishes of HEK293FT cells to 50-60% confluency in Growth media in 10cm Petri Dish.

One dish is needed to generate the GolgiTag lentivirus and the other is needed for the ControlTag lentivirus.

3 

Prepare a transfection mix to generate a lentivirus expressing the **GolgiTag** in 1.5ml Eppendorf tube containing:

- **3.8 µg pGag/Pol plasmid**
- **2.2 µg pVSVG plasmid**
- **6 µg pLJC5-KOZAK-TMEM115-3HA plasmid**
- **300 µL OptiMem**

We purify plasmids using a QIAGEN HiSpeed® Plasmid Maxi kit [Lot# 166034460] following manufactures protocols and ensure sterile reagents are used and mixtures prepared in tissue culture hood to avoid contamination.

4 

Prepare a transfection mix to generate a lentivirus vector expressing **ControlTag** in 1.5ml Eppendorf tube containing:

- **3.8 µg pGag/Pol plasmid**
- **2.2 µg pVSVG plasmid**
- **6 µg pLJC5-KOZAK-3HA-Empty plasmid**
- **300 µL OptiMem**

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We purify plasmids using a QIAGEN HiSpeed® Plasmid Maxi kit [Lot# 166034460] following manufactures protocols and ensure sterile reagents are used and mixtures prepared in tissue culture hood to avoid contamination.

5 

Prepare PEI mixture in 1.5ml Eppendorf tube.

- **40 µL 1mg/ml PEI Max 40K** dissolved in distilled water
- **600 µL OptiMem**

6  5m

Incubate each mixture (Step 3, 4, 5) separately for ~ **00:05:00** at **Room temperature** .

7 Add **310 µL PEI Mixture (Step 5)** to the **GolgiTag** in 1.5ml Eppendorf tube (Step 3) and **ControlTag** in 1.5ml Eppendorf tube (Step 4) mixtures.

8  30m

Mix and incubate each mixture at **Room temperature** for **00:30:00** .

9 Add each mixture dropwise using a P1000 sterile pipette into a single HEK293FT containing plate.

10  1d

Incubate cells **37 °C** for **24:00:00** .

11 Replace media with fresh Growth Media (discard old media) and incubate cells at **37 °C** for further **24:00:00** . 1d

12 

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Collect media that contains the lentivirus and pass through 0.45µm syringe filter. This is now the lentivirus infection media. This could be used immediately or snap frozen in liquid nitrogen and stored at **-80 °C** .

Infecting (Transducing) and Selecting cells stably expressing GolgiTag 3d

13

This is done under sterile condition in a CATEGORY 2 biological safety cabinet

14

Mix **5 mL infection media** produced in step 12 with **5 mL fresh Growth Media** (in a 15ml Eppendorf tube).

15 Add Polybrene reagent to Mix produced in step 14 using a stock of 10mg/ml dissolved in MilliQ water (which had been sterile filtered) to a final concentration of **10 microgram per milliliter (µg/mL) Polybrene** .

16 Gently add media to 10cm plate of HEK293 cells already at ~60% confluency.

Other types of mouse and human cells could be infected with this media too

17 1d

Incubate at **37 °C** for **24:00:00** .

18 1d

Change media to Growth Media and incubate **37 °C** for another **24:00:00** .

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19 To select for cells stably expressing GolgiTag or ControlTag, replace media with **10 mL freshly prepared Selection Media** .

20 After **24:00:00** , there will be observable death and a considerable number of dead floating cells will be observed. 1d

21 Change Selection Media each 24h for 3-5 days (depending on the efficiency of the transduction process). At this stage colonies of cells stably expressing the GolgiTag or empty vector should have reached 100% confluency.

22 Split cells into more dishes maintaining cells in Selection Media.

Cells should be grown only in Selection Media, going forward. Cells can be frozen down- and stored long term in liquid nitrogen. Make note in cell freezer catalogue to culture cells in Growth Media when thawed until cells are fully recovered, then grow in Selection Media.

Cell preparation for GolgiTag and ControlTag immunoprecipitation (To be undertaken 1-2 days prior to immunoprecipitation)

23 Split cells stably expressing GolgiTag or ControlTag in 15cm Petri dish. Allow to grow near confluency (approximately 24-48h depending on the seeding density).

Pre-clearing of anti-HA beads (To be done on the day of immunoprecipitation before cell lysis)

2m 30s

24

Steps should be done separately for both GolgiTag and ControlTag.

25 

Pipette **200 µL anti-HA bead slurry** into 1.5ml Eppendorf tube.

26 Insert tube containing bead anti-HA bead slurry onto a DynaMag™ Spin Magnet for 30s

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00:00:30 and carefully remove supernatant using a P1000 pipette.

27 30s

Remove bead from DynaMag™ Spin Magnet, add **1 mL ice cold KPBS** , gently pipette up and down to disperse any clumps, put on DynaMag™ Spin Magnet for **00:00:30** and carefully remove supernatant using a P1000 pipette.

28

Repeat wash step described in step 27 a further 3 times:

28.1 30s
(Wash 1/3): Remove bead from DynaMag™ Spin Magnet, add **1 mL ice cold KPBS** , gently pipette up and down to disperse any clumps, put on DynaMag™ Spin Magnet for **00:00:30** and carefully remove supernatant using a P1000 pipette.

28.2 30s
(Wash 2/3): Remove bead from DynaMag™ Spin Magnet, add **1 mL ice cold KPBS** , gently pipette up and down to disperse any clumps, put on DynaMag™ Spin Magnet for **00:00:30** and carefully remove supernatant using a P1000 pipette.

28.3 30s
(Wash 3/3): Remove bead from DynaMag™ Spin Magnet, add **1 mL ice cold KPBS** , gently pipette up and down to disperse any clumps, put on DynaMag™ Spin Magnet for **00:00:30** and carefully remove supernatant using a P1000 pipette.

29

After the last wash step, incubate the bead slurry in **50 µL KPBS** and keep beads **On ice** .

Cell lysis for immunoprecipitation

30

Steps should be done separately for both GolgiTag and ControlTag.

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31 Place cells **On ice** slighted tilted to enable medium to be easily aspirated.

32 

Remove media and immediately wash plates with **10 mL cold (4°C) PBS** .

33 Immediately, gently aspirate PBS from cells.

34 

Gently wash one more time repeating steps 32 and 33:

34.1 Remove media and immediately wash plates with **10 mL cold (4°C) PBS** .

34.2 Immediately, gently aspirate PBS from cells.

35 

Carefully remove PBS and add **1 mL cold (4°C) KPBS** .

36 Whilst cells are maintained **On ice** , scrape cells off dish with a disposable cell lifter.

37 Carefully transfer scrapped cells using an P1000 pipette into a 1.5ml Eppendorf tube.

38 

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Pellet cell by centrifugation at **1000 x g, 4°C, 00:02:00** .

39 Carefully discard supernatant using an P1000 Pipette.

40 Resuspend pellet in **950 µL ice cold KPBS** .

41 Pipette cells up and down with a P1000 pipette ensure cells are in suspension and remove **50 µL** into a 1.5 ml Eppendorf tube marked as "**cell lysate INPUT**". Keep this **On ice**

- Place the tube containing the remaining ~900µl of resuspended cells on ice and take sample in the cold room.

Remaining steps of this section and all of the next section to be undertaken in cold room.

42 

Pipette the **900 µL cells** into a clean 2ml Dounce homogeniser.

43 Homogenise cells with exactly 25 strokes, making sure bubbles are avoided.

- To verify efficacy of homogenisation, take 10µl on a slide, briefly check under the microscope. There should be about at least 80% cell lysis, indicated by the visible presence of nuclei without surrounding cytoplasm.

If cells are not sufficiently lysed undertake another 10 strokes of homogenisation and check for cell lysis a second time-repeat as necessary but avoid over homogenisation, to maintain integrity of organelles.

44 

Centrifuge lysate at **1000 x g, 4°C, 00:02:00** . This is to remove any non-lysed cells, nuclei and debris such as cell membranes.

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45 Remove supernatant (which should contain organelles) into a new 1.5 ml Eppendorf tube. The lysate at this stage will be between 700-800µl.

46 Remove **50 µL** from the homogenate into a separate 1.5ml Eppendorf labelled as **IP INPUT**. Keep **On ice** .

GolgiTag and ControlTag immunoprecipitation 17m

47

This must be done in a cold room.

48 Take out Eppendorf tube containing the washed anti-HA beads from step 29.

49 Add the cell homogenate from step 45 into the eppendorf tube containing the HA-beads.

50 

Ensure that bead clumps are dispersed by gently pipetting up and down 5 times using a P1000 Pipette.

51  5m

Incubate the cell homogenate-HA-bead slurry for **00:05:00** on orbiter.

52 Separate bead by putting tube on DynaMag™ Spin Magnet for **00:00:30** . 30s

53 Collect **50 µL** of supernatant into separate 1.5ml Eppendorf tube label as **FT Supernatant (FT = flow-through)**. Keep **On ice** .

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54 Wash bead with 1000 µl of ice cold KPBS 3 times:

54.1 (Wash 1/3): Wash bead with **1000 µL ice cold KPBS** .

54.2 (Wash 2/3): Wash bead with **1000 µL ice cold KPBS** .

54.3 (Wash 3/3): Wash bead with **1000 µL ice cold KPBS** .

55 Incubate beads with **500 µL ice cold KPBS** and prepare a slurry by pipetting up and down 5 times with a P1000 pipette and transfer slurry into a clean 1.5 ml Eppendorf tube.

This step is important as it ensure contamination from cell extract sticking to tube plastic is minimized.

56 Insert tube containing anti-HA bead slurry onto a DynaMag™ Spin Magnet for **00:00:30** 30s and carefully remove supernatant using a P1000 pipette.

57 Add **80 µL ice-cold lysis buffer** to the HA beads containing the immunoprecipitated Golgi.

Depending on application different solubilization buffers could be deployed at this stage.

58 10m

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Incubate for **00:10:00** **On ice** .

59 Place tube on DynaMag™ Spin Magnet for **00:00:30** to separate beads. 30s

60 Collect supernatant into new Eppendorf tube.

This is important to avoid contamination.

61 To ensure total removal of beads, add a further **20 µL ice-cold lysis buffer** to HA-Beads and place the beads again on DynaMag™ Spin Magnet for **00:00:30** and collect supernatant into Eppendorf tube containing the solubilized Golgi in step 60. 30s

62 

Determine the protein concentration of the IP using a BCA Protein Assay Kit. Use **5 µL solubilised Golgi** (do not dilute) for quantification purpose. Quantification should be done in duplicate.

63 

Depending on future application samples can be snap frozen in liquid nitrogen and stored at **-80 °C** or diluted into 4xLDS loading buffer supplemented with fresh 5% (by vol) 2-mercaptoethanol prior to analysis on SDS-polyacrylamide gel electrophoresis and immunoblot analysis. For Immunoblot purpose, 2-4 µg of solubilised Golgi is enough to detect Golgi markers (GM130, ACBD3 and GOLGIN97).

Preparation of INPUT sample 20m

64 

Pellet cells by centrifugation at **1000 x g, 4°C, 00:02:00** .

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65 Discard supernatant and resuspend cell pellet in **80 µL ice-cold lysis buffer** .

66 

Pipette up and down 10-20 times with a P200 pipette.

20m

67 

Incubate lysate **On ice** for **00:20:00** .

68 

Centrifuge lysate at **13000 x g, 4°C, 00:10:00** .

69 

Collect clarified cell lysate supernatant in fresh tube and determine protein concentration using a BCA Protein Assay Kit. Dilute an aliquot of the supernatant in lysis buffer at a ratio 1:10, before protein quantification.

70 Depending on future application the clarified cell lysate can be snap frozen in liquid nitrogen and stored at **-80 °C** or diluted 4X into 4xLDS loading buffer supplemented with fresh 5% (by vol) 2-mercaptoethanol prior to analysis on SDS-polyacrylamide gel electrophoresis and immunoblot analysis.

Preparation of Flow-through and IP INPUT sample 20m

71 Add **100 µL ice-cold lysis buffer** to tubes marked **FT** and **IP INPUT**.

72 

Mix thoroughly by pipetting up and down using P1000.

20m

73 

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Incubate **On ice** for **00:20:00** .

74

Centrifuge lysate at **13000 x g, 4°C, 00:10:00** .

75

Collect clarified cell lysate supernatant in fresh tube and determine protein concentration using the BCA method. Dilute an aliquot of the supernatant in lysis buffer at a ratio 1:10, before protein quantification.

76 Depending on future application the clarified cell lysate can be snap frozen in liquid nitrogen and stored at **-80 °C** or diluted 4X into 4xLDS loading buffer supplemented with fresh 5% (by vol) 2-mercaptoethanol prior to analysis on SDS-polyacrylamide gel electrophoresis and immunoblot analysis.

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