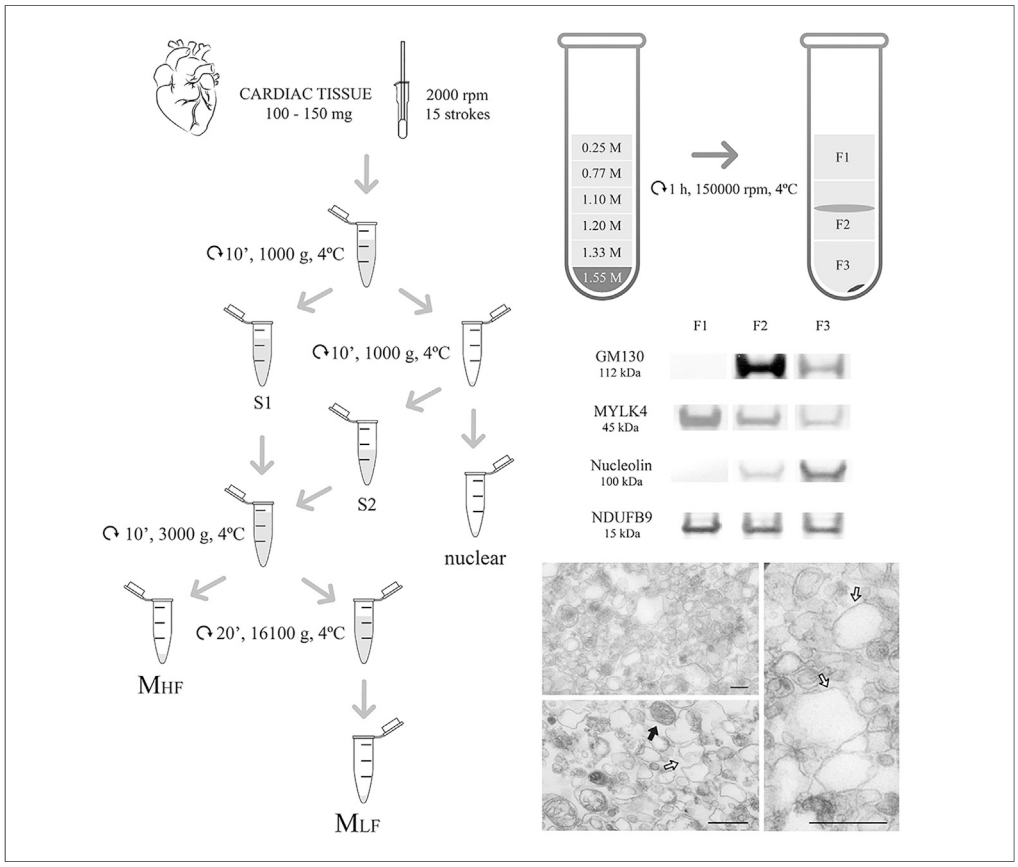
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for Isolation of Golgi Vesicles from Human and Animal Hearts by Flotation through a Discontinuous Sucrose Gradient



Detailed study of cellular organelles requires their isolation. Several protocols have been described for the isolation of the Golgi apparatus from liver tissue, but these are not suitable and not reproducible in harder tissues. Here, we describe a protocol to isolate Golgi vesicles from cardiac tissue using a discontinuous sucrose gradient.

Estefanía Tarazón, Manuel Portolés, Esther Roselló-Lletí

tarazon_est@gva.es (E.T.)
esther_rosello_lleti@hotmail.com (E.R.-L.)

HIGHLIGHTS

Protocol for isolating Golgi vesicles using a discontinuous sucrose gradient

Isolating Golgi vesicles from cardiac tissue for proteomics and other applications

Golgi vesicle analysis provides mechanistic insight into disease pathophysiology

Protocol also allows isolation of nuclear and mitochondrial fractions for various use

Tarazón et al., STAR Protocols *1*, 100100
September 18, 2020
https://doi.org/10.1016/j.xpro.2020.100100

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for Isolation of Golgi Vesicles from Human and Animal Hearts by Flotation through a Discontinuous Sucrose Gradient

Estefanía Tarazón,[1,2,*] Manuel Portolés,[1] and Esther Roselló-Lletí[1,3,*]

[1]Myocardial Dysfunction and Cardiac Transplantation Group and CIBERCV, Health Research Institute of La Fe University Hospital (IIS La Fe), Valencia 46026, Spain

[2]Technical Contact

[3]Lead Contact

*Correspondence: tarazon_est@gva.es (E.T.), esther_rosello_lleti@hotmail.com (E.R.-L.)
https://doi.org/10.1016/j.xpro.2020.100100

SUMMARY

Detailed study of cellular organelles requires their isolation. Several protocols have been described for the isolation of the Golgi apparatus from liver tissue, but these are not suitable and not reproducible in harder tissues. Here, we describe a protocol to isolate Golgi vesicles from cardiac tissue using a discontinuous sucrose gradient.

For complete details on the use and execution of this protocol, please refer to Tarazon et al. (2017).

BEFORE YOU BEGIN

⚠ CRITICAL: All solutions, materials, and equipment should be precooled to 0°C to 4°C and kept on ice throughout. Centrifuge rotors should be precooled to the same temperature.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Sucrose	Sigma-Aldrich	S1888
Tris-Cl 1 M pH 7.4	Sigma-Aldrich	T2194
Critical Commercial Assays		
Total Protein Kit, Micro Lowry, Peterson's Modification	Sigma-Aldrich/MERCK	TP0300-1KT
Antibodies		
GM130 primary rabbit monoclonal antibody	Abcam	ab52649
Nucleolin primary mouse monoclonal antibody	Merk Milipore	05-565
MYLK4 primary rabbit polyclonal antibody	Abcam	Ab107994
NDUFB9 primary rabbit polyclonal antibody	Sigma-Aldrich	SAB1101190
Rabbit secondary Alkaline Phosphatase antibody	Sigma-Aldrich	A3687

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse secondary Alkaline Phosphatase antibody	Sigma-Aldrich	A3562
Biological samples		
100 to 150 mg of fresh or frozen heart tissue samples	n/a	n/a
Other		
Sterile Surgical Blades No.22	Braun	n/a
Scalpel handle No.4	Braun	n/a
Potter-Elvehjem PTFE pestle and glass tube, working volume 3 mL	Sigma	P7734
Cole-Parmer™ Stirrer Motor with Electronic Controller, Cole-Parmer™ 04369-25	Fisher Scientific	117660058
Eppendorf™ 5415R Refrigerated Centrifuge	Sigma-Aldrich	Z605212
Optima™ L-100 K Preparative Ultracentrifuge with a Type 70.1 Ti Fixed-Angle Titanium Rotor	Beckman Coulter	393254 and 342184
10 mL open-top thickwall polycarbonate tubes	Beckman Coulter	355630

Alternatives: This protocol is optimized using the resources specified in above Key Resources Table but they are not exclusive products to carry it out. It is highly recommended optimizing the protocol first if any resources are modified because there is no guarantee that any change could compromise the results.

MATERIALS AND EQUIPMENT

- Homogenization medium: add 17.2 g sucrose (0.25 M final), and 2 mL 1 M Tris-Cl 1 M pH 7.4 (10 mM final) up to 200 mL H_2O. Adjust to pH 7.4 if required. Store at 4°C for up to 1 to 2 days.
- Tris-Cl 10 mM: add 500 µL Tris-Cl 1 M to 49.5 mL H_2O. Adjust to pH 7.4 if required.
- 2 M stock and sucrose gradient solutions preparation according to Table 1. Store up to 1 to 2 days at 4°C.

Note: The sucrose concentration in each gradient solution is very important for the correct isolation of Golgi vesicles, so it is recommended to verify this concentration using a refractometer.

For additional reagents and equipment necessary to protein concentration determination, western blot and immunohistochemistry, and transmission electron microscopy analysis, please refer to Roselló-Lletí et al. (Rosello-Lleti et al., 2015).

Table 1. Sucrose Gradient Solutions Preparation

	2 M	1.33 M	1.2 M	1.1 M	0.77 M
Tris-Cl 1 M (mL)	1	n/a	n/a	n/a	n/a
Sucrose (g)	69	n/a	n/a	n/a	n/a
Sucrose 2 M (mL)	n/a	16.63	15	13.75	9.63
H_2O (mL)	up to 100	n/a	n/a	n/a	n/a
Tris-Cl 10 mM (mL)	n/a	8.38	10	11.25	15.38

n/a, not applicable

STEP-BY-STEP METHOD DETAILS

This protocol is a modification of ones described in Graham, 2001 and Graham and Winterbourne, 1988 meant to make the protocol suitable for harder tissues. If the protocol is performed with human samples, the written informed consent of each participant in the study must be obtained, and the investigation must comply with the principles outlined in the Declaration of Helsinki (Macrae, 2007). If the protocol is carried out with animal samples, the investigation must conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and European (2010/63/EC, if that is the case) and the National (the one that corresponds) Directive. Furthermore, in both cases the project must be approved by the Biomedical Investigation Ethics Committee of the institution where it is carried out.

Note: The time of each step will increase depending on the number of samples to extract. Isolation of Golgi vesicles of a large number of samples at the same time is not recommended to avoid waiting times during homogenization, and degradation. Depending on the experience of the researcher, the isolation of four samples at the same time could be optimal.

Note: The protocol shown below is described point by point performed with human heart tissue; however, initially the method was developed with rat heart tissue. Slight variations were found that have been reflected in the protocol in Problems and Potential Solutions.

Human Cardiac Tissue Homogenization

Timing: 30 min

1. Cut up 100–150 mg of cardiac tissue (fresh or freeze) into small pieces (1 mm^3) at 4°C or on dry ice.

⚠ CRITICAL: The protocol can be performed immediately after processing the samples (fresh tissue) or using samples that have been previously frozen at −80°C. In any case, the time between obtaining the sample and freezing at −80°C or performing the protocol should be minimized, being preferable that it is immediate and always keeping the sample at 4°C. Samples require additional cutting into small pieces to ensure proper homogenization. In both cases, place a sterile petri dish on a flat surface of ice (for cutting fresh samples) or dry ice (for cutting frozen samples) and put in 100–150 mg of heart tissue. Use a sterile blade and a clean scalpel handle to cut samples into small pieces of approximately 1 mm^2. Special care must be taken with the frozen samples since their hardness makes the cutting more difficult and the sample must be prevented from thawing. Use sterile 1,000 μL pipette tips to hold them and make cuts very slowly, as a quick cut can cause the sample to "jump" and get lost.

2. Suspend mince in homogenization medium (4 μL × each mg tissue) and transfer this suspension to the glass vessel of a Potter-Elvehjem homogenizer.
3. Attach the cold homogenizer pestle (from Potter-Elvehjem) to an electric motor and homogenize cardiac mince at 2,000 rpm with 15 strokes of the pestle. The motor should be fixed on a stable surface that prevents it from swinging, using a G clamp.
4. Transfer the homogenate to a 1.5 mL Eppendorf tube and wash the pestle with same volume per sample (4 μL × each mg tissue) of homogenization medium to remove any adhering residual tissue. Retrieve the volume in the same 1.5 mL Eppendorf tube.

Prepare Light Mitochondrial Fraction

Timing: 1 h 30 min

5. Centrifuge for 10 min at 1,000 × *g* at 4°C in an Eppendorf™ 5415R Refrigerated Centrifuge to pellet nuclei.

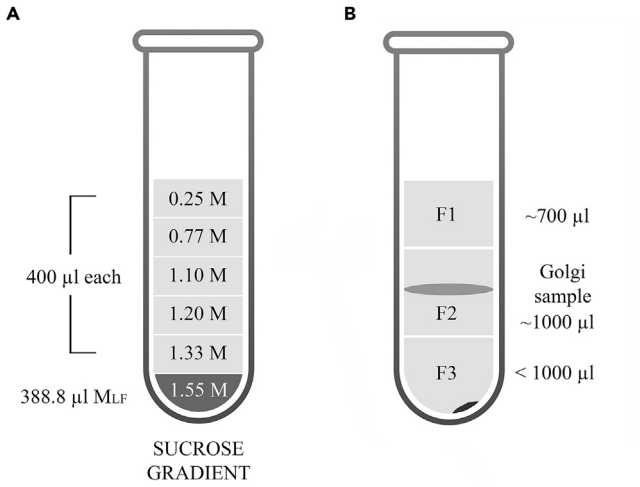


Figure 1. Vesicle Golgi Separation by Sucrose Gradient Ultracentrifugation
(A) Sucrose gradient.
(B) Isolated fractions.

6. Transfer the supernatant to a clean tube and put it on ice (S1, reserve).
7. Resuspend the nuclear pellet with a pipette in homogenization medium (2 µL × each mg of tissue), and centrifuge for 10 min at 1,000 × *g* at 4°C (Eppendorf™ 5415R Refrigerated Centrifuge).
8. Combine the supernatant from step 6 (S1) with the latter supernatant step 7 (S2) and centrifuge for 10 min at 3,000 × *g* at 4°C (Eppendorf™ 5415R Refrigerated Centrifuge) to pellet the heavy mitochondrial fraction (MHF)
9. Transfer supernatant to a clean 1.5 mL Eppendorf tube and centrifuge 20 min at 16,100 × *g* at 4°C (Eppendorf™ 5415R Refrigerated Centrifuge).
10. Aspirate supernatant and discard, without disturbing the pellet, light mitochondrial fraction (MLF).

Isolate Golgi-Derived Vesicles

Timing: 2 h 30 min

11. Resuspend the MLF pellet in 100 µL of homogenization medium, then add 288.8 µL of sucrose 2 M (final concentration ≈ 1.55 M) and mix gently.
12. Check that the suspension forms a layer under the 1.33 M sucrose solution, as shown in Figure 1A.

Pause Point: MLF suspension can be frozen and the protocol may continue at another time. The samples will be frozen at −20°C if continued the next day or kept at −80°C if the period of time is longer.

13. Transfer all volume (388.8 µL) of MLF to a 10 mL ultracentrifuge tube and overlay it with the following sucrose gradient solutions: first 400 µL sucrose 1.33 M, followed by the same volume of 1.2 M, 1.1 M, 0.77 M and finally 0.25 M; depositing each solution of the gradient with great care, to avoid mixing between them, along the wall of the tubes with tips of 1,000 µL (Figure 1A).

Note: To maintain better control of the solution deposit, it is not recommended to cut the tips of 1,000 µL. After all solutions are deposit, there should be six phases that are visible when the tube is held up to the light (Figure 1A).

14. Centrifuge in an Optima™ L-100 K Preparative Ultracentrifuge (Beckman Coulter) for 1 h at 150,000 × *g* at 4°C.

Harvest Golgi Vesicles

Timing: 45 min

15. Harvest material that bands at the upper half of the tubes that corresponds to 0.77 M/1.1 M and 1.1 M/1.2 M sucrose interfaces and any intervening material. Specifically, the tube can be divided into three fractions: the top (about 700 μL, F1), the half (about 1 mL, F2) and the bottom (remainder, less than 1 mL, F3). In half (F2), a band corresponding to the Golgi vesicles sample should be observed (Figure 1B).

Note: Visually, although with difficulty, the layers of the gradient can still be seen and a cloudier band that corresponds to the sample. To collect the phases, the first phase including 0.25 M layer and part of 0.77 M is carefully taken with a 1,000 μL tip; next the second phase including the rest of the 0.77 M layer, the 1.1 M layer and the 1.2 M layer is taken; finally the third phase including from the 1.33 M layer to the bottom is taken.

16. Dilute each fraction with 1 volume of homogenization medium (1 mL) and centrifuge for 20 min at 16,100 × *g* at 4°C (Eppendorf™ 5415R Refrigerated Centrifuge).
17. Remove F2 supernatant and resuspend the pellet in 20 μL of homogenization medium to determine protein concentration or continue with fixing to electron microscopy analysis.

Note: This pellet should be visually observed as a small, diffuse pellet. A protein concentration of 5 ± 2 μg/μL would be expected but it will depend to a great extent on the characteristics of the tissue used, especially the degree of fibrosis.

Pause Point: Golgi vesicles samples can be long-term stored at −80°C and used for different purposes. It is recommended to determine the protein concentration before use and not before storage.

Protein Determination

The protein content of the suspensions were determined by Peterson's Modification (Winters and Minchin, 2005) of the Lowry method using Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma-Aldrich/MERCK) following manufactured instructions.

EXPECTED OUTCOMES

Eukaryotic cells are organized into compartmentalized organelles with specialized functions exerted by distinct sets of proteins, lipids, and metabolites. The structure, composition, or function of organelles might be masked by the cellular or tissue complexity under study or by their low abundance. Therefore, isolation of key organelles would allow more efficient identification and study of their function or the intrinsic molecules of interest.

Vesicular trafficking is a cellular process that regulates numerous basic and specialized functions required to sustain life. The molecular machinery and regulation of cellular processes involving vesicular trafficking are highly complex. Because of this and because these processes are intimately interconnected with a broad number of cellular functions, vesicular transport is susceptible to alterations in many disease conditions. Explore the potential changes in these routes is significant in order to understand their pathological implications and contribution to disease outcomes.

Currently, the most cellular models available do not properly reflect key physiological parameters of the biological environment in the body. Therefore, the analysis of the isolated vesicles of the

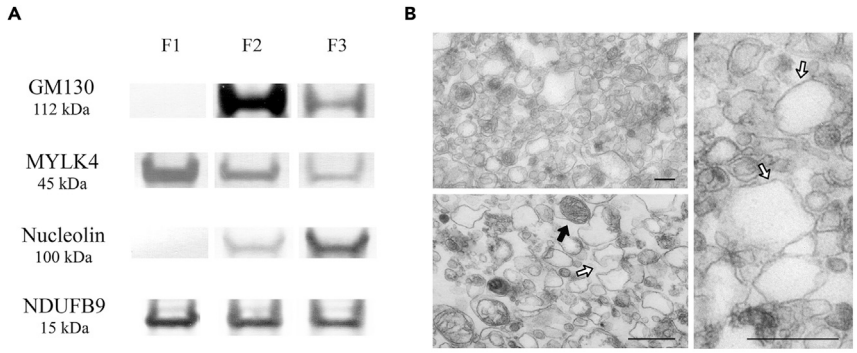


Figure 2. Analysis of Isolated Fractions

(A) Western blot of Golgi (GM130 and MYLK4), nucleus (nucleolin and MYLK4), cytoplasmic (MYLK4), and mitochondrial (NDUFB9) markers in the three fractions collected after the sucrose gradient ultracentrifuge. F1, fraction 1 (top); F2, fraction 2 (half); F3, fraction 3 (bottom).

(B) Visualization of isolated Golgi vesicles using transmission electron microscopy (white arrows). Numerous smooth vesicles are seen, mostly formed by a single membrane, apparently empty with little dense content, mainly a light network of granular material. The presence of mitochondrial remains is also observed (black arrow). Bar represents 500 nm.

Golgi apparatus, both morphologically, as well as their composition or function, can provide us valuable information on the pathophysiological mechanisms of the disease under study. In this sense, this protocol aims to isolate Golgi vesicles from cardiac tissue for use in a number of downstream applications. One of the most important applications of subcellular fractionation and protein enrichment is in proteomics, where a simplification of the complex protein mixtures facilitates its analysis. These analyses allow obtaining information on key biological processes under various cellular conditions. But also, they can be used in other applications such as 2D/MS, electron microscopy, disease profiling, protein expression and interaction, and enzymatic or localization studies.

According to the isolation process, it is expected to find these vesicles at the sucrose interfaces corresponding to 0.77 M/1.1 M and 1.1 M/1.2 M (F2, Figure 1B). At this fraction, a diffuse band containing Golgi vesicles will be observed. By western blot analysis of the fractions, performed as we described in Roselló-Lletí et al (Rosello-Lleti et al., 2015) to GM130 (1/500), MYLK4 (1/100), nucleolin (1/250) and NDUFB9 (1/250) (Figure 2A), it is confirmed that the F2 fraction is the one that contains the greatest amount of pure Golgi apparatus vesicles. Thus, in F2 the presence of a greater amount of established Golgi GM130 marker is observed as well as MYLK4. Furthermore, the nuclear part is deposited in F3, although it will not be possible to avoid finding some contamination in F2, in the same way that occurs with the mitochondrial fraction that is concentrated in F1 (Figure 2A).

Additionally, electron microscopy, performed as we described in Roselló-Lletí et al. (Rosello-Lleti et al., 2015) and observed with a JEOL JEM-1010 electron microscope with magnifications ranging from ×3,000–12,000, shows the quality of the isolated vesicles (Figure 2B). The material obtained in this isolation is similar to the vesicles observed in the isolates described by Fleischer et al. (Fleischer et al., 1969) in bovine liver and Ehrenreich et al. (Ehrenreich et al., 1973) in rat liver. They are numerous smooth vesicles mostly formed by a single membrane, apparently empty with little dense content, mainly a light network of granular material. This material appears to be sufficiently to warrant subsequent chemical and enzymological studies.

Finally, this protocol isolates different fractions (nuclear, heavy mitochondrial and light mitochondrial fractions) that may be of interest to the researcher.

LIMITATIONS

Golgi vesicles purification from microsomal fraction may be problematic since these vesicles will be both denser and lighter than some of the other populations, and fragments of endoplasmic reticulum will be frequently isolated together. However, in the light mitochondrial fraction Golgi is the least dense component of this fraction. Therefore, the isolation is less problematic, but in this fraction some endoplasmic reticulum may continue being present. Similarly, as can be seen in the western blots, there will be a certain presence of nuclear and mitochondrial fraction. This is the main limitation to take into account since, although we selected the fraction with the greatest number of pure Golgi vesicles, as many purity checks should be made as the researcher needs.

On the other hand, the experience of the researcher will play a very important role in minimizing the processing times, avoiding degradation, and preserving the quality of the samples.

TROUBLESHOOTING

Problem 1

Non-homogenized tissue remained and could be observed in step 3 and may require additional homogenization if the protein concentration obtained does not reach the minimum amount necessary for the purpose of isolation, taking into account the percentage of fibrosis of the tissue used and the expected average concentration described in step 17. It is possible that the tissue does not homogenize with 2,000 rpm and 15 strokes.

Potential Solution

Within a range of 500 to 2,800 rpm, the 2,000 rpm speed was the best performer but can be adapted to the available tissue. Similarly, in a range of 10 to 20 strokes, 15 strokes provided the best results.

Problem 2

When performing an SDS-PAGE and a Coomassie blue staining, the different bands are not observed throughout the gel or they are diffuse, which would indicate that protein degradation is occurring during isolation.

Potential Solution

It is necessary to take into account the cold preservation of the tissue throughout the process, avoiding overheating, especially if the speed or the number of strokes is increased during homogenization. Working at low temperatures at all times, keeping the samples on ice, dry ice or at 4°C, as the case may be, helps with the folding and stability of the proteins, as well as slows down the activity of the proteases that can be released in the isolation process. Also, the faster the protocol steps are performed, the less time they have to interact and possibly degrade proteins. Therefore, it is vitally important to carry out the isolation process as quickly as possible and at low temperatures, adapting it to each step of the protocol. If necessary, protease inhibitor can be added to all solutions, considering that in many cases it can interfere with later experiments.

Problem 3

The band of the Golgi vesicles is not observed after ultracentrifugation (step 15).

Potential Solution

The amount of starting tissue is very important; an amount close to 150 mg gives better results in a range of 50 to 150 mg. However, the availability of cardiac tissue may be a limitation. Therefore, the volume of resuspension of the MLF pellet indicated in the step 11 can also be important. Within a range of 50 to 200 μL of homogenization medium, sometimes using 50 μL may be the best choice. In any case, the sucrose volume must be adjusted at the same time to obtain a final concentration of 1.55 M.

Problem 4

Pellet of the Golgi vesicles are not observed after final centrifugation in step 16.

Potential Solution

The sucrose concentration in this final centrifugation of step 16 is crucial for the precipitation of Golgi vesicles. Dilution of the F2 with one volume of homogenization medium (1 mL) is usually sufficient but samples can be diluted with two volumes of this buffer (2 mL) to improve the results. In addition, checking the sucrose concentration of the solutions using a refractometer as indicated can be very helpful.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Esther Roselló-Lletí (esther_rosello_lleti@hotmail.com).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

This study did not generate or analyze datasets or code.

ACKNOWLEDGMENTS

This work was supported by the National Institute of Health "Fondo de Investigaciones Sanitarias del Instituto de Salud Carlos III" (PI16/01627, PI17/01925, PI17/01232, CP18/00145), "Consorcio Centro de Investigación Biomédica en Red, M.P." (CIBERCV, under grant CB16/11/00261), and the European Regional Development Fund (FEDER). We are grateful to Dr. Raquel Cortés of the Genotyping and Genetic Diagnosis Unit of the INCLIVA Biomedical Research Institute, the Microscopy Unit of the Polytechnic University of Valencia, and the Microscopy Unit of the IIS La Fe for their technical support in this work. The authors are grateful to Mr. Arash Javadinejad, English Instructor and Publication Editor at the Health Research Institute La Fe, Valencia, Spain, for professional English language editing.

AUTHOR CONTRIBUTIONS

Conceptualization, E.T.; Investigation, E.T.; Writing – Original Draft, E.T.; Writing – Review & Editing, E.T., E.R.L., and M.P.; Funding Acquisition, E.R.L. and M.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Ehrenreich, J.H., Bergeron, J.J., Siekevitz, P., and Palade, G.E. (1973). Golgi fractions prepared from rat liver homogenates. I. Isolation procedure and morphological characterization. J. Cell Biol. *59*, 45–72.

Fleischer, B., Fleischer, S., and Ozawa, H. (1969). Isolation and characterization of Golgi membranes from bovine liver. J. Cell Biol. *43*, 59–79.

Graham, J.M. (2001). Isolation of Golgi membranes from tissues and cells by differential and density gradient centrifugation. Curr. Protoc. Cell Biol., Chapter 3, Unit 3 9.

Graham, J.M., and Winterbourne, D.J. (1988). Subcellular localization of the sulphation reaction of heparan sulphate synthesis and transport of the proteoglycan to the cell surface in rat liver. Biochem. J. *252*, 437–445.

Macrae, D.J. (2007). The council for international organizations and medical sciences (CIOMS) guidelines on ethics of clinical trials. Proc. Am. Thorac. Soc. *4*, 176–178, discussion 178-9.

Rosello-Lleti, E., Tarazon, E., Barderas, M.G., Ortega, A., Molina-Navarro, M.M., Martinez, A., Lago, F., Martinez-Dolz, L., Gonzalez-Juanatey, J.R., Salvador, A., Portoles, M., and Rivera, M. (2015). ATP synthase subunit alpha and LV mass in ischaemic human hearts. J. Cell Mol. Med. *19*, 442–451.

Tarazon, E., Rosello-Lleti, E., Ortega, A., Gil-Cayuela, C., Gonzalez-Juanatey, J.R., Lago, F., Martinez-Dolz, L., Portoles, M., and Rivera, M. (2017). Changes in human Golgi apparatus reflect new left ventricular dimensions and function in dilated cardiomyopathy patients. Eur. J. Heart Fail. *19*, 280–282.

Winters, A.L., and Minchin, F.R. (2005). Modification of the Lowry assay to measure proteins and phenols in covalently bound complexes. Anal. Biochem. *346*, 43–48.