Myelinosome-like vesicles in human seminal plasma A cryo-electron microscopy study

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Title: Myelinosome-like vesicles in human seminal plasma: a cryo-electron microscopy study

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Key words : Extra cellular vesicles; seminal plasma; human; myelinosome; Sertoli cells

Abbreviations

EV: extra cellular vesicles; **cryo-EM**: cryo electron microscopy; **TEM:** transmission electron microscopy**, MVB :** multivesicular bodies.

Abstract

Seminal plasma is particularly rich in extracellular vesicles. Myelinosomes are membranous organelles described throughout the seminiferous epithelium of the testis but never reported in semen. Our aim was to determine the presence of myelinosomes in human seminal plasma.

Transmission electron microscopy and cryo electron microscopy analysis of standard myelinosome preparation from TM4 Sertoli cells and human seminal plasma samples.

We have specified by cryo-EM the morphological aspect of "standard" myelinosomes isolated from the culture media of TM4 Sertoli cells. Vesicles with the same morphological appearance were revealed in human seminal plasma samples.

Human seminal plasma contains a population of large EV (average diameter 200 nm) whose morphological appearance resemble those of myelinosomes. Defining the specific biomarkers and functionalities of myelinosome in human seminal plasma are the concerns to be addressed in our further research.

INTRODUCTION

Seminal plasma is a complex fluid produced by the secretions from all organs of seminal tract [15]. Being an environment for the male gametes, seminal plasma is enriched in extracellular vesicles (EVs). They are produced in the male reproductive tract, by the accessory sex glands, including seminal vesicles and prostate, as well as by the epididymis, therefore EVs from seminal plasma are also called prostasomes or epididymosomes. The term "prostasome" concerns a heterogenous population of bilayered EVs found in seminal plasma of various species [1]. Structural heterogeneity and protein composition of these exosome-like vesicles has been shown in human semen [9]. EVs from seminal plasma are known to participate in various aspects of male fertility, improving spermatozoa function through regulation of the timing of sperm cell capacitation, induction of the acrosome reaction, stimulating sperm motility to attain fertilizing capacity. Other proposed functions of prostasomes include interfering with the destruction of spermatozoa by immune cells within the female reproductive tract [1]. Indeed, it has been observed that there are significantly improved outcomes when women are exposed to seminal plasma [4].

EVs fraction consists of heterogeneous population of extracellular vesicles. The particles of human seminal plasma were first classified according morphological criteria, including multiplicity of vesicles, their shape, their external protrusions and their overall density [9]. According these features, three types of EV in human seminal plasma were defined. In recent years, the development of new technologies in Electron Microscopy have led to an increase of knowledge with a finer and more specific analysis for all types of EVs. A recent study extended the data revealing 5 major subcategories of EVs and 6 subcategories of extracellular membrane compartments, including lamellar bodies [7].

Myelinosomes are membranous organelles found in the seminiferous epithelium of the testis [6;16] and seemingly secreted by the somatic Sertoli cells in the lumen of the seminiferous tubules [10]. It has been suggested that Sertoli cells get rid of misfolded proteins through myelinosomes-mediating secretion, maintaining thereby cell proteostasis [16]. In transmission EM micrographs, myelinosomes are recognized as markedly electron-dense osmiophile membraneous vesicles, showing a stacked, reticulated or whorled arrangement.

The aim of this study was to look for the presence of myelinosome vesicles in human seminal plasma. Because of the viscosity of seminal gel and its water-holding capacity, classical transmission electron microscopy does not seem to be an optimal technique to reveal the presence of myelinosomes in this fluid. Cryo-electron microscopy is a technique that allows visualization of nanosized structures without prior fixation or addition of heavy metals for contrast. The sample is therefore visualized as close to its native state as possible [8]. Therefore, using standard myelinosome preparation from TM4 Sertoli cells, we first analyzed the appearance of "standard" native myelinosomes by cryo EM and then compared it with the vesicles from human seminal plasma samples.

MATERIALS AND METHODS

Cells and cell transfection protocol

Murine TM4 Sertoli cells were from IGBMC (Strasbourg, France) cell culture service collection. TM4 Sertoli cells were cultured in DMEM/F12 medium (Gibco, number 31330), containing 2.5% FBS, 5% HS, and supplemented with 1% antibiotic mix (10 000 U/ml penicillin and 50 mg/ml streptomycin, GIBCO 15140). $0.5x10^6$cells were plated into 6-well dishes and cultured at 37°C with 5% CO_2. Cells were tested for mycoplasma contamination using mycoplasma detection kit (MycoAlert, LONZA LT07-418).

Transient transfection of TM4 cells with expression vectors pEGFP-C2-trHtt-142Q generated at IGBMC (Strasbourg, France) were performed using Lipofectamine 2000 (Invitrogen) reagent in 6-well plates cells following the manufacturer's instructions. The ratio of plasmid/Lipofectamine in 2.0 ml of serum-free incubation mix was 4.4µg/16µl.

Standard myelinosomes from TM4 Sertoli cells.

Myelinosomes were isolated from culture media of TM4 Sertoli cells transiently transfected with plasmid vector coding for mutant Huntingtin-exon1-EGFP protein as previously described [16]. Briefly, 48 h post-transfection the myelinosomes were purified from the collected culture medium as follows: the cultured medium was centrifuged for 10 min at 600g. The pellet was discarded and the supernatant centrifuged for 90 min at 20 000g. The supernatant was discarded and the pellet obtained contains the fraction enriched in

myelinosomes. 20 000g myelinosome-enriched fraction also contained 100-120 nm microvesicles. The pellet was suspended in a culture media for immediate use.

Human samples

Human samples were provided by the GERMETHEQUE BIOBANK. Semen from 4 healthy donors was collected and left to liquefy at room temperature for 30 min. Cells and large pieces of undissolved seminal gel were removed by centrifugation at 800g for 20 min. The supernatant was centrifuged at 100 000g for 1 hour, and the pellet was used for cryo-electron microscopy.

Cryo-electron microscopy

Vitrification of the samples was performed using an automatic plunge freezer (EM GP, Leica) under controlled humidity and temperature [5]. The samples were deposited to glow-discharged electron microscope grids followed by blotting and vitrification by rapid freezing into liquid ethane. Grids were transferred to a single-axis cryo-holder (model 626, Gatan) and were observed using a 200 kV electron microscope (Tecnai G2 T20 Sphera, FEI) equipped with a 4k × 4k CCD camera (model USC 4000, Gatan). Micrographs were acquired under low electron doses using the camera in binning mode 1 and at a nominal magnification of 25,000x.

RESULTS:

In total, images of 289 slices were analyzed: 100 from TM4 cells as references and 189 from human seminal plasma.

1) Analysis of TM4 Sertoli cell secretion

Using standard myelinosome preparation from culture media of TM4 Sertoli cells expressing mutant Huntingtin-exon1-EGFP protein, we analyzed the morphological aspect of secretory myelinosome vesicles by TEM and Cryo electron microscopy. Figure 1a & b (left panel) shows the electron micrograph of myelinosome vesicles by TEM technique. Myelinosome

vesicles are electron-dense osmiophile particles, which consist of numerous whorled membranes forming the regular stacks. Because of sectioning of myelinosome preparation during TEM processing, some of myelinosomes exhibit an interior cavity, filled with amorphous material, the others seem to be “intact”. Higher magnification of such “intact” myelinosome allows discerning the stacks of electron-dense membranes, which seem to be whorled around a central small vesicle, which is also formed of few membrane layers. Standard preparations of myelinosome vesicles also contain several EVs, which are clearly discerned in TEM micrographs. Figure 1 (right panel) shows the preparation of myelinosomes vesicles analyzed by Cryo-Tem technique. Because Cryo-TEM technique does not require sample sectioning, only “intact” myelinosomes can be seen in Cryo-TEM micrographs. High magnification allows discerning the regular stacks in myelinosome vesicle, as well as a central small vesicles, likewise as those seen in TEM preparation (Figure 1a & 1b). Both TEM and Cryo techniques revealed a structural identity of myelinosome vesicles (electron density, whorled organisation of membrane layers, distance between membrane layers).

Comparing to TEM samples, more heterogeneity in the morphology of small EVs were observed in the samples processed by Cryo-TEM technique. Indeed, in Cryo-TEM micrographs myelinosome vesicles were surrounded by EVs, which were often unequal in regard of their sizes and shapes (round, elliptic).

2) Analysis of human seminal plasma samples by Cryo-TEM

The viscosity of seminal gel and its water-holding capacity are limiting factors impeding a good resolution by TEM technique, but not by Cryo-TEM. Careful examination of samples from human seminal plasma by Cryo-TEM revealed the presence of unusual vesicles, whose size and morphology resembled those of myelinosomes from TM4 secretions (Figure 2a &2b). Indeed, comparing with neighboring EVs, these unusual vesicles were electron dense and contained multiple membrane layers. In some cases the small central vesicles were discerned in central part of these organelles, likewise as in secretory myelinosomes from TM4 Sertoli cells (Figure 1). Nevertheless, comparing to “standard” myelinosomes, the unusual vesicles from human seminal plasma contained fewer membrane layers and exhibited lesser density of membrane packaging. Remarkably, such vesicles have not been described in previous studies [7;9]. Intracellular myelinosomes from different cells and/or species are

known to exhibit a huge variability of forms by TEM analysis. No such information was available for the secretory myelinosomes, organelles recently described [16]. Thus, it seemed likely that the secretory myelinosomes could also present the variability of morphological organization, including the density of membrane packaging.

3) Comparative analysis of EVs from human seminal plasma and secretion products from TM4 Sertoli cells

In preparations from both human seminal plasma and culture media of TM4 Sertoli cells, myelinosomes vesicles were present together with other EVs. Remarkably, the vesicles from both species shared striking similarities. Indeed, in both preparations we observed heterogenous membrane-bound vesicles some of them containing amorphous material (Figure 3). The particles generally varied in size from 50 to 500 nm. Morphologically, some structures resemble multivesicular bodies, specialized forms of endosomes involved in exocytosis. Exocytosis is believed to be one of the mechanisms that promote EV release from prostatic epithelial cells. Light EV appear to be analogous to intraluminal vesicles released during exocytosis (Figure 3). Some vesicles and membrane compartments showed a more electron dense interior, indicative of a lumen filled with cargo. We have seen electron density inside double vesicles, triple or more vesicles. Sporadic multilayered membrane complexes with large concentric membrane fragments were also visualized (Figure 4). Sporadic small tubules were also observed in the preparations from both human seminal plasma and from the secretory products of TM4 Sertoli cells (Figure 5). Main types of EVs from both sources (human seminal plasma and secretory products from TM4 Sertoli cells) are summarized in Table 1 based on the classification of Polyakov *et al*. [9]. According this classification, 3 main class of EVs are present in human seminal plasma. Because the secretory myelinosomes and myelinosome-like vesicles have not been reported earlier, we added them in the table as the fourth class of EVs.

DISCUSSION

Here we show for the first time the presence of myelinosome-like organelle in human seminal plasma. Secretory myelinosome is a new type of extracellular vesicle, which has been found *in vitro* in culture media from murine TM4 Sertoli cells, expressing aggregate-prone proteins mutant Huntingtin and F508delCFTR (cystic fibrosis transmembrane modulator). While *in situ* secretory myelinosomes were detected in the lumen of seminiferous tubules from R6/2 HD model mice [16], the presence of myelinosome vesicles in human biological fluids have never been reported.

In TEM micrographs intracellular myelinosomes are discerned as 200-500 nm osmiophile vesicles, consisting of whorled membrane layers, which can be easily distinguished on the cross-sections. Intracellular myelinosomes exhibit a huge variability of forms in TEM. According our data "standard" secretory myelinosomes from TM4 cells also revealed a morphological variability in their form, as well as in the number and density of membrane layers.

To our knowledge, no cryo-TEM examination of conventional and secretory myelinosomes have been undertaken up to now. We specified by cryo-TEM the morphological aspect of "standard" secretory myelinosomes emitted by TM4 Sertoli cells. By cryo-TEM technique secretory myelinosome is as round-shaped or elliptic electron-dense vesicle, consisting of stacked membranes. Thus, both TEM and cryo-TEM techniques seem to be quasi equal for revealing of myelinosomes in biological samples.

Myelinosome-like vesicles we found in human seminal plasma by Cryo-TEM technique fitted the morphological criteria determining "standard" myelinosome. Indeed, round-shaped vesicles, formed with whorled membranes, exhibited strong electron density. Comparing to myelinosome from TM4 Sertoli cells, myelinosome-like vesicle from seminal plasma looked more friable and seemed to contain fewer membrane layers. The latter is difficult to assess because no sectioning can be provided by Cryo-TEM technique.

Preparations from both human seminal plasma and secretions products of TM4 Sertoli cells also contained a heterogeneous population of EVs with different morphological aspect. In a recent study, Hoog and Lotvall [7] showed that in human ejaculate, about 59% of EVs are found to be single vesicles, while the rest are oval vesicles, double vesicles, double special vesicles, triple vesicles, tubules or lamellar bodies. This morphological variability may reflect

different subpopulations exerting different functions in physiological and pathological processes. As believed, these structurally specific EVs originate from several male sexual organs (prostate, epididymis, testis, seminal vesicles). Remarkably, our data indicate that nearly all types of secretory product we observed in human seminal plasma were also present in the secretions from TM4 Sertoli cells. This suggests an important contribution of testicular secretion in the formation of EV pool of seminal plasma. The former was traditionally underestimated, and the idea on the main role of prostate gland in the production of EVs in seminal plasma was prevalent.

Our data indicate that in both types of samples the single exosome-like vesicles predominated and constituted about 50% of EV population. These exosome-like EVs often surrounded myelinosomes in the preparations of human seminal plasma and TM4 Sertoli cell culture media. Of note is that the intracellular traffic and excretion of myelinosomes by TM4 Sertoli cells is supported by multivesicular bodies (MVB), which are implicated in the transport of various specific organelles in other cell types [2;14]. TM4 Sertoli cells secrete myelinosomes together with the exosomes following the fusion of MVBs with plasma membrane [16]. Therefore, the presence of exosome-like EVs in close proximity of myelinosomes in human seminal plasma specimens suggests the MVB-related mechanism of their release in seminal fluid. Remarkably, “standard” myelinosome vesicles emitted from TM4 Sertoli cells are devoid of molecular markers of exosomes/prostasomes as CD63 protein [16].

It seems plausible that not only Sertoli cells, but also several cell types of the male reproductive tract contribute to the production of myelinosomes. Indeed, the epithelial cells of prostate gland and epididymis are characterized by high secretory activity, being responsible for the production of prostasome and epididymosome EVs for seminal fluid through apocrine and merocrine mechanisms. These EVs containing both DNA [11] and RNA [13] are important for acquisition of the sperm-fertilizing ability and sperm forward motility, for the protection of sperm against reactive oxygen species [12]**.** Prostasomes also have immunosuppressive activity via protection against complement attack, and have a role in the in the stabilization of the sperm plasma membrane [11].

To date no information on the specific markers of myelinosomes as well as on their physiological role in the human seminal fluid is available. In aggregation diseases such as HD

or cystic fibrosis, myelinosome vesicles seem to be the protective particles providing isolation of cells from potentially toxic aggregate-prone proteins. On the other hand, being absorbed by neighboring cells, myelinosomes vesicles, carrying the pathological proteins can contribute to pathological spreading. We are only in the beginning of knowledge about myelinosome vesicles. To date we only note their presence in human seminal plasma, and upcoming studies would bring the information about their role and significance for human fertility.

CONCLUSION

EVs have the capacity to convey information between cells through the transfer of functional protein and genetic information to recipient cells associated with reproductive biology. In the male reproductive tract, epididymosomes and prostasomes participate in regulating sperm motility activation, capacitation, and acrosome reaction [3]. Here we evidence a new type of EVs, namely a myelinosome-like vesicle in the human seminal plasma. It remains to define the specific biomarkers and physiological functionalities of this organelle still poorly known.

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Contributions

M.Y. and C.R. conceived the study and wrote the manuscript, M.Y. and AS.N. realized the preparation of samples, B.J. and N.B. critically revised the manuscript. All authors have approved the final manuscript.

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Legends of figures:

Fig.1: Preparations of “standard” myelinosome fraction from TM4 Sertoli cells by TEM (a & b; b is a selection from a) and Cryo-TEM (c & d; d is a selection from c) techniques.

Fig.2: Preparations human seminal plasma by Cryo-TEM techniques.

Fig.3: Heterogeneity of EVs in seminal plasma (left) and in the secretion products from TM4 Sertoli cells (right).

Fig.4: Pleiomorphic aspect of EV from human seminal plasma and from secretion products released by TM4 Sertoli cells.

Fig.5: Non-vesicular constituents of human seminal plasma (a,b,c) ; tubules observed and secretion products released by TM4 Sertoli cells (d, e, f,g).

Legends of the table:

Table 1: description of EV in seminal plasma according modified Poliakov’s classification [9].

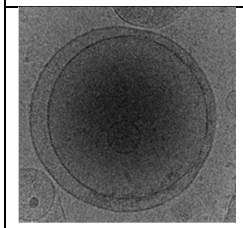
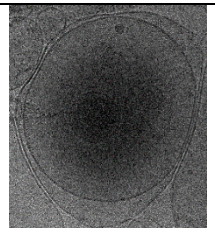
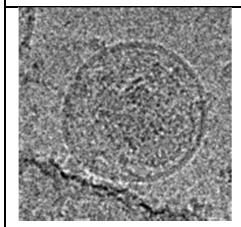
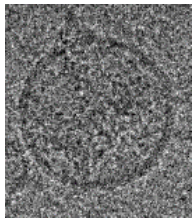
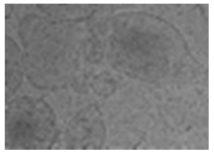
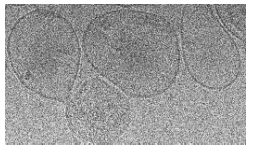
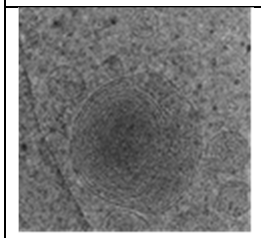
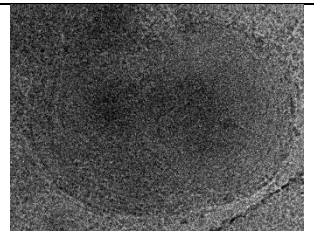
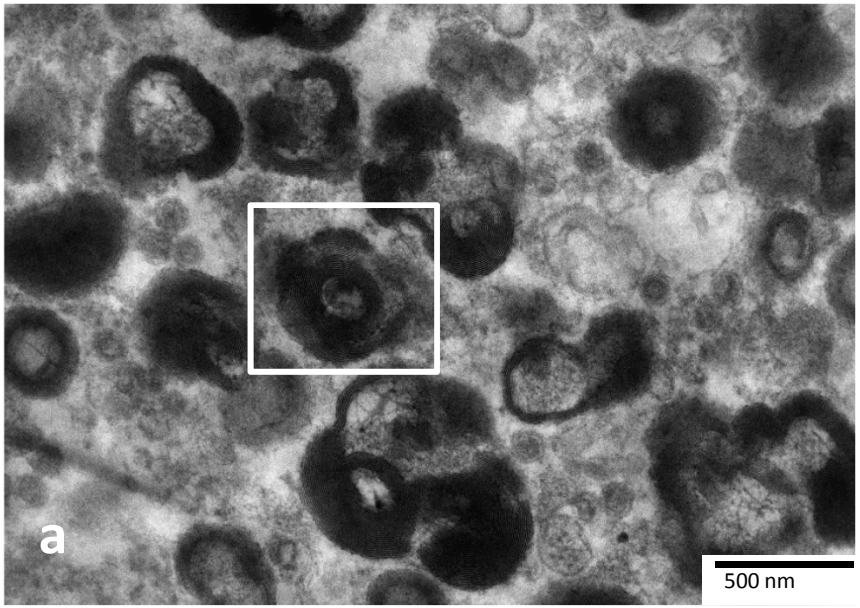
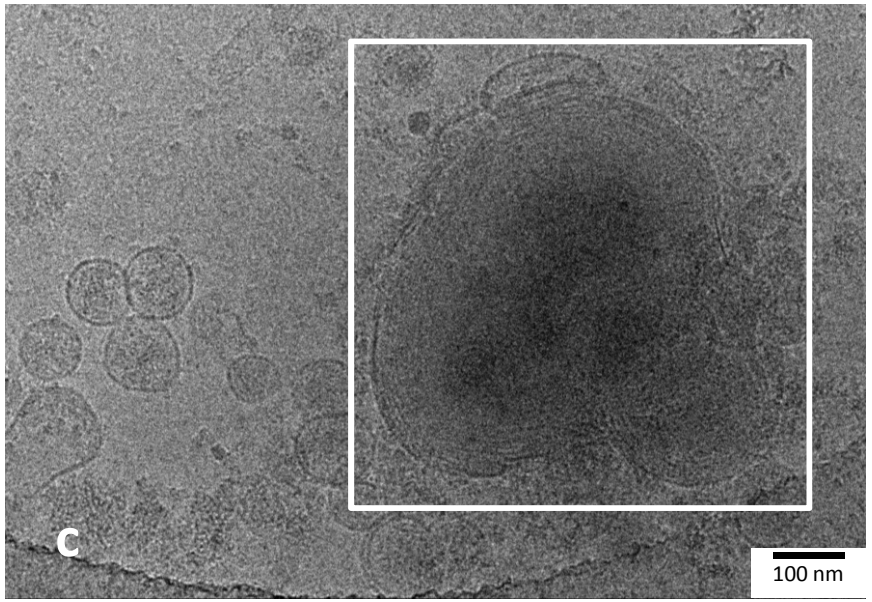
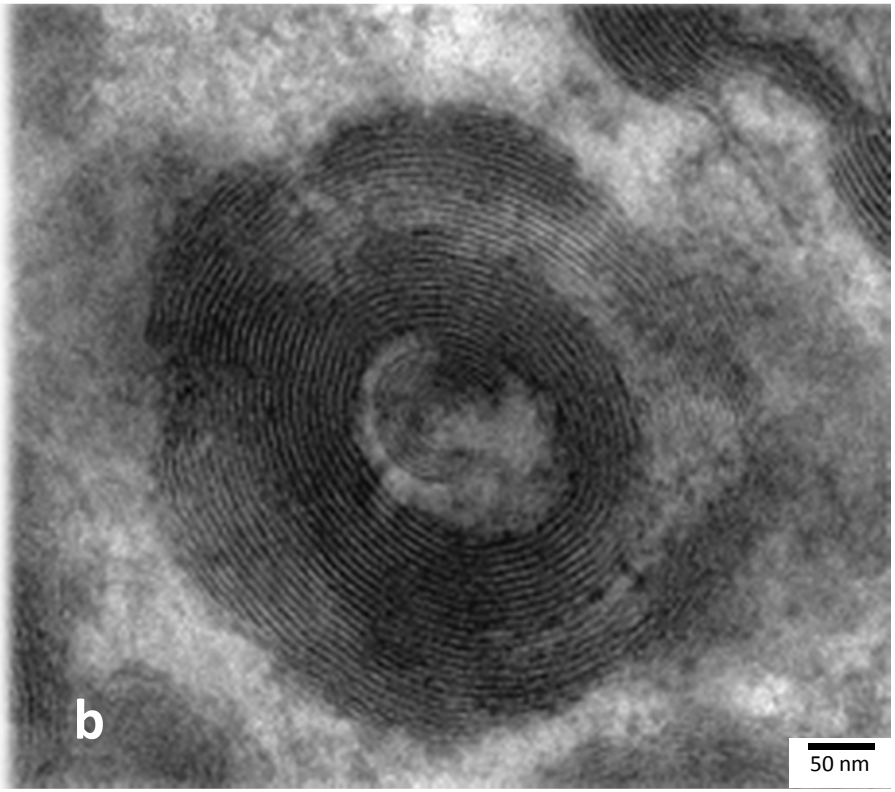
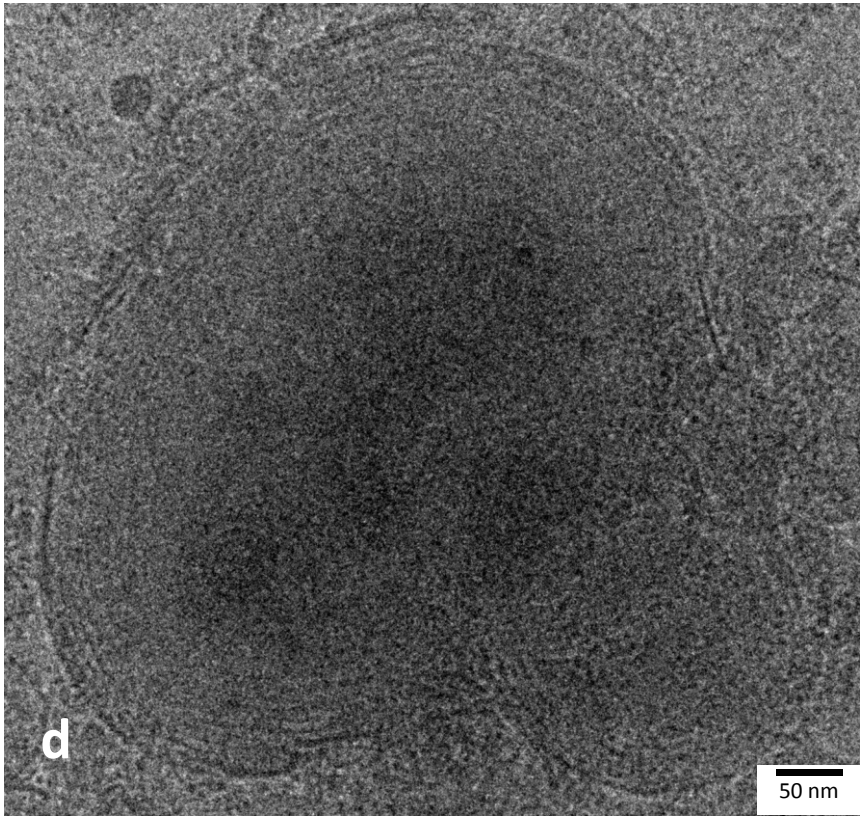
Human seminal plasma	EV secreted by murine TM4 Sertoli cells	Type	Description	Size	Fraction in the
		Type 1	Dark vesicles High electron density vesicles with asymmetric deposition of dark substance surrounding one or more secondary vesicles. These particles are invariably smooth on the outside, lacking protrusions, and are nearly round or slightly elongated.	from 100 to 200 nm.	around 50%.
		Type 2	Light vesicles Low electron density vesicles with abundant protrusions. These vesicles do not have internal depositions of dark substance, but sometimes contain daughter vesicles, and are nearly round or only slightly elongated.	from 50 to 200 nm.	25–30% of all v
		Type 3	Low electron density vesicles without protrusion features, presenting as elongated, or pear-shaped, with no dark depositions, and smooth outer surface.	they can be as long as 400 nm and about 50–100 nm wide	20–25% of the
		Type 4	Myelinosomes round-shaped vesicles, formed with whorled membranes, exhibited strong electron density	200-700 nm	<1%

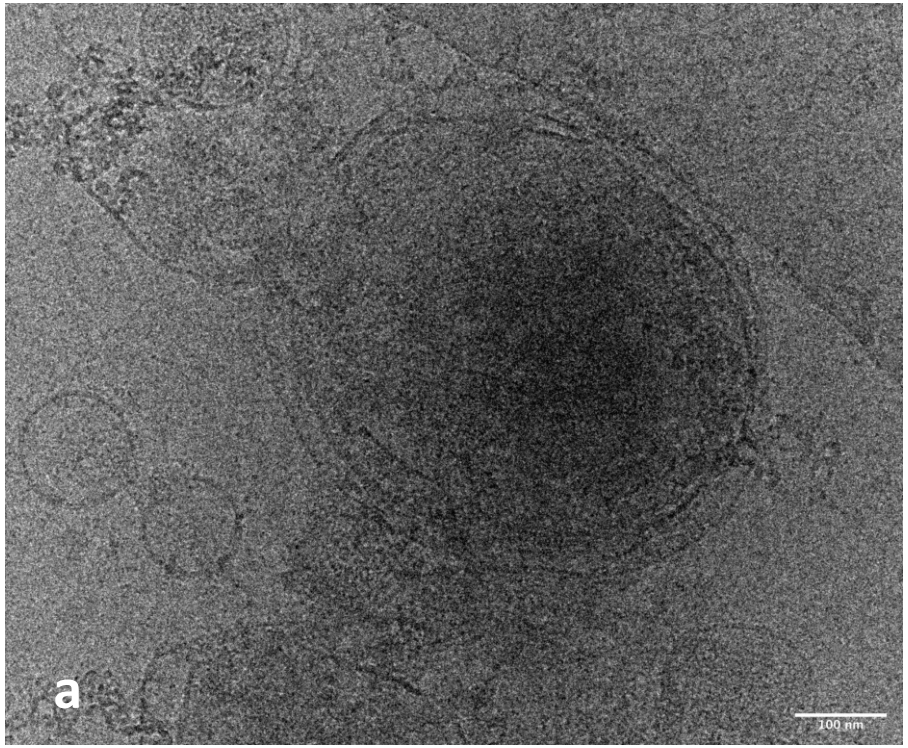
Table 1: description of EV in seminal plasma according modified Poliakov's classification [9].

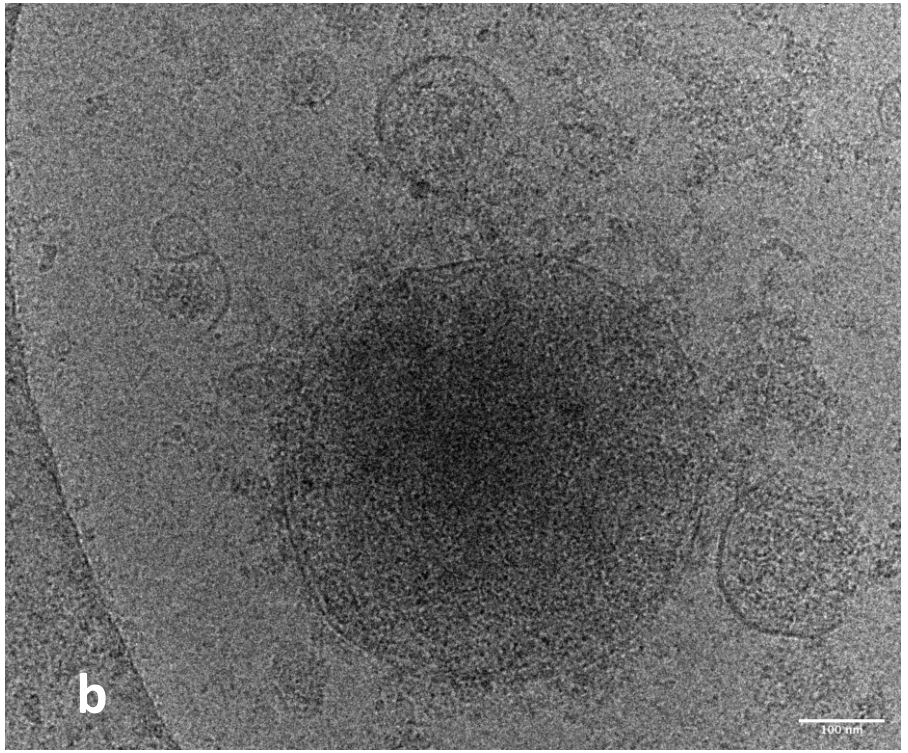

a
500 nm

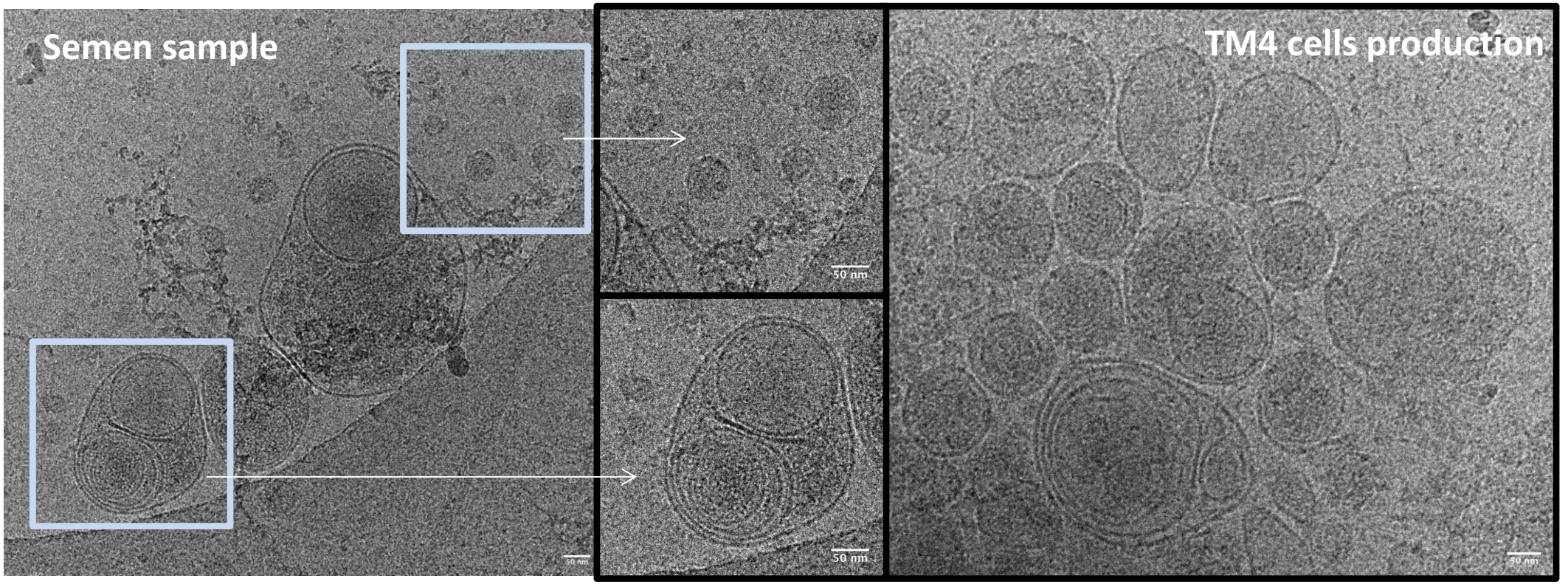

c
100 nm

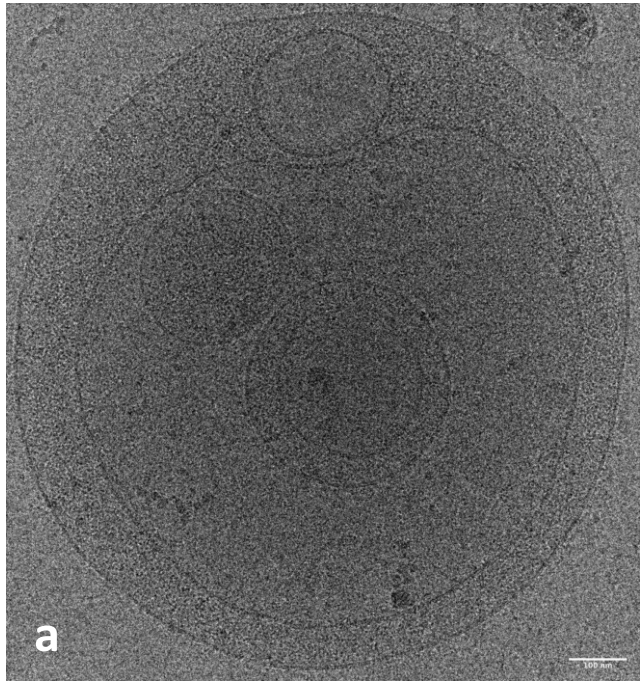

b
50 nm

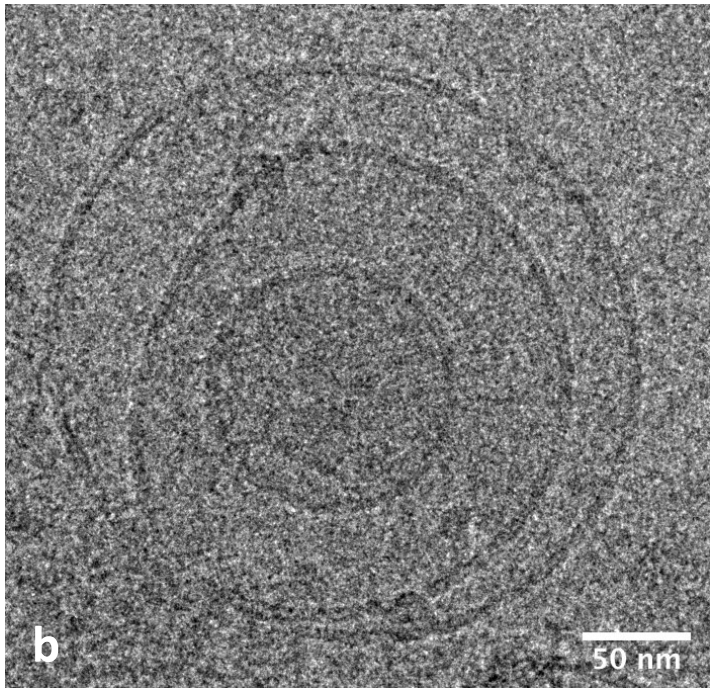

d
50 nm

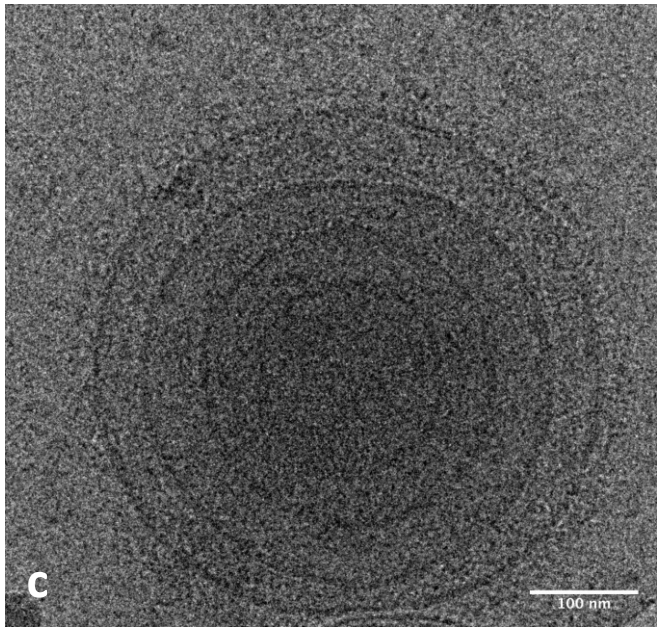

a
100 nm

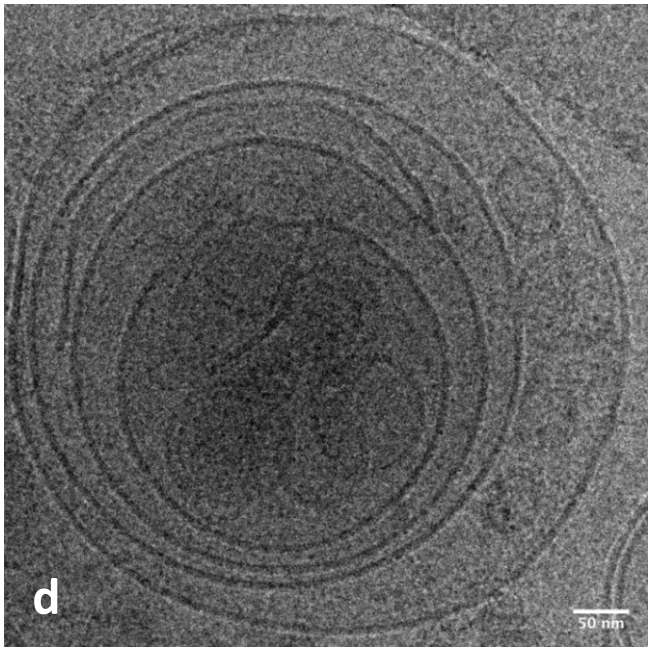

b
100 nm


Semen sample
50 nm
50 nm
50 nm
TM4 cells production
50 nm


a


b
50 nm


c
100 nm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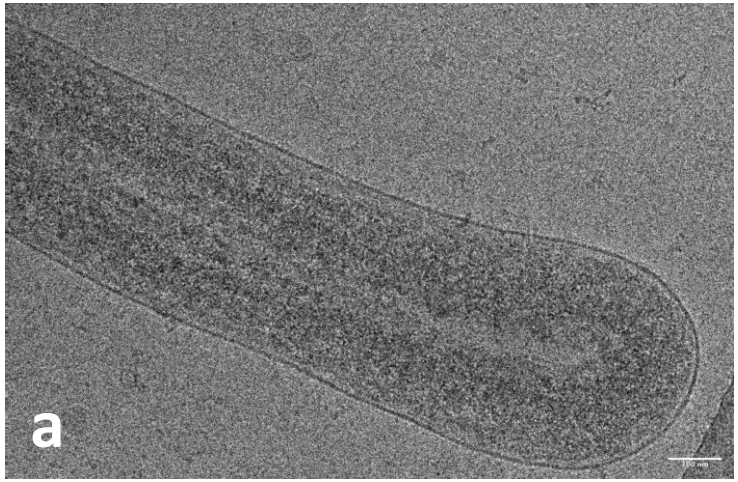
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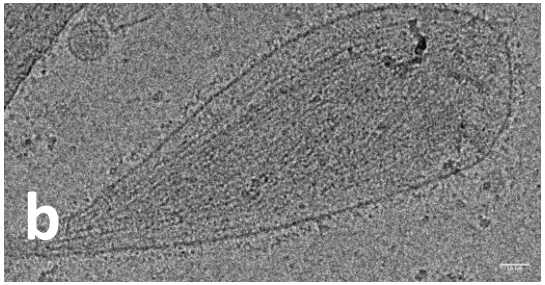
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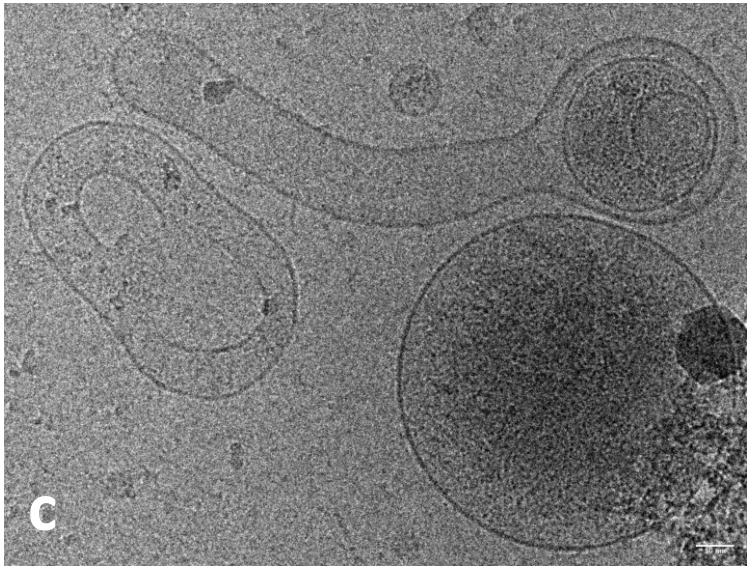
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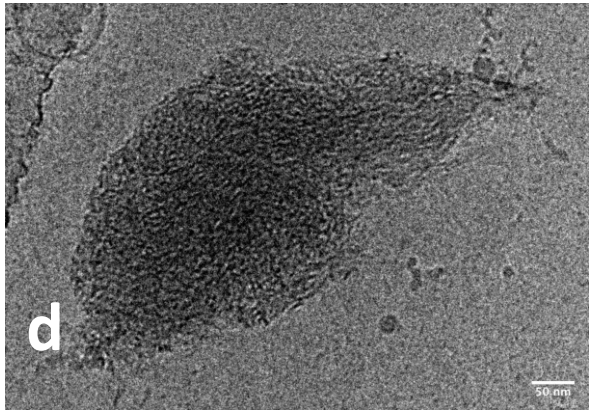
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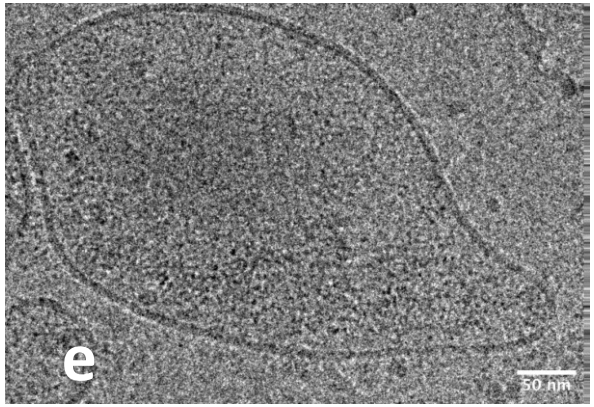
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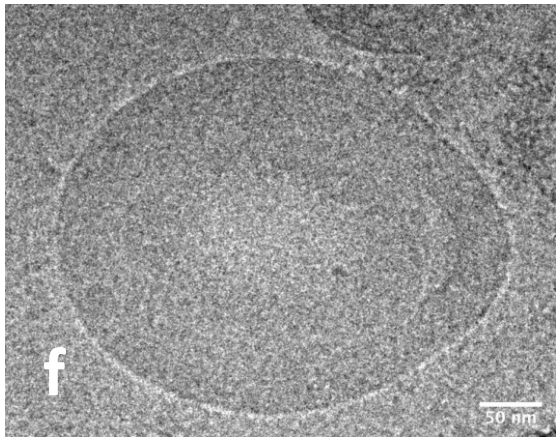
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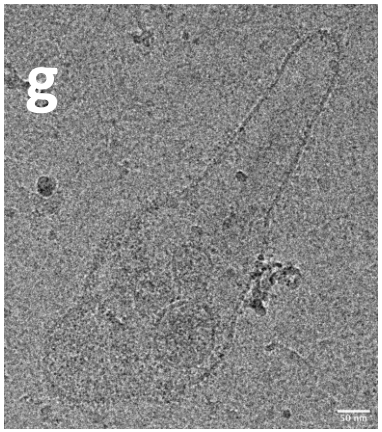
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