Extended Abstract

Protocol optimization for the ultrastructural preservation of cilia in *Drosophila melanogaster's* antenna

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Abstract

Cilia are microtubule-based organelles involved in a variety of processes, such as sensing, motility and cellular architecture-organizing functions. Moreover, they are altered in several human conditions called ciliopathies and are involved in cancer. To understand these pathologies, a detailed knowledge of the biology of cilia is required. These organelles are remarkably well conserved throughout eukaryotic evolution and have been well studied in *Drosophila melanogaster (Dm)*.

Dm is an advantageous model organism to study several biological and physiological properties since they are conserved between the fly and mammals, and nearly 75% of human disease-causing genes are believed to have a functional homolog in the fly. Other advantages are the availability of powerful genetics tools, highly conserved disease pathways, very low comparative costs, rapid life cycle and no ethical problems.

Despite these advantages, there is still a gap in the application of transmission electron microscopy (TEM) to study the ultrastructure of *Dm*. One of the reasons is the difficult-to-process chitin exoskeleton that surrounds the fly body, in particular the antenna, an organ that contains ciliated neurons. Also, basic sample preparation procedures for resin embedding of biological specimens have not evolved much since the 60's and newer methods such as cryo techniques have not been used to study this organ.

Therefore, the goal of this project is to have an optimized TEM protocol, both for chemical and cryo processing in *Dm*'s antenna, which can help to provide the necessary leap to expand the utilization of *Dm*'s antenna in research.

KEY-WORDS: Ultrastructure, protocol optimization, cilia, *Drosophila melanogaster*, chemical fixation, cryo fixation.

Introduction

Cilia are 9-fold symmetric microtubule-based structures that protrude from the cell membrane. They can be motile or immotile and they have several functions in the cells such as allowing motility (in the sperm flagellum), move particles around (in the respiratory epithelium) and responding to various external signals (intercellular communication). The cilium has two compartments: (1) the transition zone, linked to a basal body docked to the cell membrane and (2) the axoneme (Jana et al, 2014).

At the transition zone (TZ) the doublet microtubules are heavily cross-linked to the surrounding ciliary membrane by structures called y-linkers. The TZ may act as a gatekeeper for material that goes

into cilia. The doublet microtubules of the TZ are thought to be a template for the microtubule skeleton of the cilium, (the axoneme), which consequently exhibits a 9-fold symmetry (Carvalho-Santos, 2012).

Since cilia are a component of most eukaryotic cells, when these organelles are altered they can lead to a wide variety of human diseases, which are referred to as ciliopathies. These diseases present a large spectrum of conditions including various syndromes, sterility, microcephaly, *situs inversus*, polycystic kidney disease, retinal degeneration, and dwarfism (Waters, A. Beales, P., 2011). Also, alterations of these organelles have been linked to cancer. All of these implications caused by cilia alterations stress the need of having a better understanding of cilia biology and structure.

All structures described above were initially studied using conventional transmission electron microscopy, revealing remarkable features and structural complexity (Jana et al, 2014). Much more can be studied and learned using both the conventional electron microscopy approach and cryo techniques for tissue fixation.

To carry out these techniques, model organisms need to be used. Since cilia are conserved eukaryotic organelles several different model can be used such as *Drosophila melanogaster* (*Dm*) (Jana et al, 2014). *Dm* has several ciliated cells such as ciliated sensory neurons in the antenna (Vincensini, 2011). The antenna is caped with an exoskeleton, the cuticle, which contains lipids and polysaccharide chitin. It serves as a protective barrier and as an interface with the environment (Boseman et al, 2013). Chitin is a tough material that forms a diffusion barrier against fixative and other chemicals, making chitin rich samples difficult to fix (McDonald, 2012). Even so, some studies on the antenna of *Dm* have been done using chemical fixation proving that it is possible to preserve such structure if an optimized protocol is used (Todi et al, 2004; Jana, 2011).

However, chemical fixation very commonly introduces artifacts in the samples. If the goal of a researcher is to do ultrastructural studies of a specific organelle or cellular structure, these artifacts can decrease the strength of the results obtained with this technique.

To overcome this problem, and to achieve a greater level of precision, cryo fixation techniques can be used. This method usually produces improved structural preservation when compared to chemical fixation methods (McDonald, 2014).

Several cryo-fixation techniques were adapted for *Dm* tissues (McDonald, 2012; 2014). However, so far there are no reports in the literature of a cryo-fixation study that focuses on the antenna of the *Dm*, in particular the third segment.

Therefore, the goal of this study was to create an optimized protocol that preserved the ultrastructure of the antenna of *Dm*. To do so, this study focused on the more classical electron microscopy approach, chemical fixation and on the more recent technology for optimized ultrastructural preservation, cryo fixation.

Materials and Methods

An evaluation table to quantitatively assess the ultrastructural quality of samples was created. This table allows the comparison between variables in the same protocol and the comparison between different protocols. In this table, the ultrastructural preservation of several cell components is evaluated (cellular membrane, nuclear membrane, cytoplasm, intercellular space, mitochondria and cilia) and also the general preservation of the tissue and the absence of artifacts. The final score of a sample can vary from zero to one, zero being the worst and one being the best score.

For chemical fixation five different buffers (Phosphate, PHEM, PBS, Cacodylate and Water) were tested. Flies were put to sleep with CO_2 and the heads were removed from the fly. The heads were immersed in fixative (2% formaldehyde, 2.5% glutaraldehyde in 0.1 M buffer) pH 7.4 for 30 min. The third antennal segments were pierced using a thin tungsten needle. Heads were transferred to the same fixative and were fixed overnight at 4ºC with rotation. Samples were washed for 5 x 5min in the buffer used for the fixative solution. Post-fixation was performed in 1% Osmium tetroxide (OsO_4), for 1hr30min at 4ºC. Samples were washed with Milli-Q water for 5 x 5min. The samples were incubated in 2% uranyl acetate for 20 min in RT with rotation. Samples were washed in Milli-Q water 3 × 10 minutes. The samples were dehydrated in a graded series of alcohol (50%, 70%, 90% and 3x 100%) for 10 min in each solution. Incubation with propylene oxide was done for 2 x 15 minutes with rotation followed by incubation with 1:1 propylene oxide: EPON resin for 3 hrs. The samples were infiltrated with EPON resin overnight at 4ºC with rotation. The next day, the samples were transferred to fresh resin for 2 hrs at RT with rotation.

For cryo fixation three freeze substitution (FS) durations (Fast-6hr30min, Medium-19hr30min and Slow-52hr) were tested. Flies were put to sleep with CO_2 and the heads were removed from the fly. The heads were immersed in fixative (2% formaldehyde, 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4) for 30 min. The third antennal segments were pierced using a thin tungsten needle and were transferred to the same fixative and were fixed for another 15 min. The samples were transferred to a 0.150µm thick aluminum carrier previously dipped in 1-hexadecene and containing 10% BSA in phosphate buffer. The sandwich was closed with a flat carrier and transferred to a carrier holder. The carrier holder was loaded in the HPF machine and the sample was frozen (at -90ºC with 2100 bar). The carrier holder was transferred to a small box filled with liquid nitrogen. When the automatic freeze substitution machine was at the right temperature (-90ºC) the carriers were transferred into a custom made metallic support. The carriers were opened and transferred into a plastic support that has specific places to keep the carrier throughout sample processing. The freeze substitution cocktail (1%OsO4, 0.5% UA and 5% water in acetone) was cooled to -90ºC and added to the samples. The samples sat in the cocktail for 3 hr (fast FS), 10hr (medium FS) or 36hr (slow FS). The temperature increased until 0º over 3hr (fast FS), 9hr (medium FS) or 15hr30min (slow FS). Washing in fresh acetone was done at 0º for 1hr30min. Embedding was done in a graded series of acetone and Epon resin: 50% Epon in resin, 100% Epon for 30 min each and new 100% Epon.

All the samples (chemical and cryo fixed) were aligned in the molds with resin and labeled by a colleague external to the study. The sample number was randomized and during the entire imaging and analysis the technician was completely blinded. Only after the scoring of all the samples the identity of the samples was revealed to the analyzer. Serial thin sections (70 nm) were cut in a Leica Reichert Ultracut S ultramicrotome, collected on formvar-coated copper slot grids, and stained with uranyl acetate and lead citrate (Hayat, 1989). Samples were examined and photographed at 100kV using a Hitachi 7650 TEM. A panel of six representative pictures of the areas to be studied was acquired per condition. These panels were used for the quantitative analysis of the sample using the evaluation table for judging ultrastructure quality. Statistical analysis on the sample scores was performed using the non parametric Kruskall-Wallis test and Dunn's multiple comparisons test.

Results

Using unbuffered fixatives reduces the quality of ultrastructural preservation

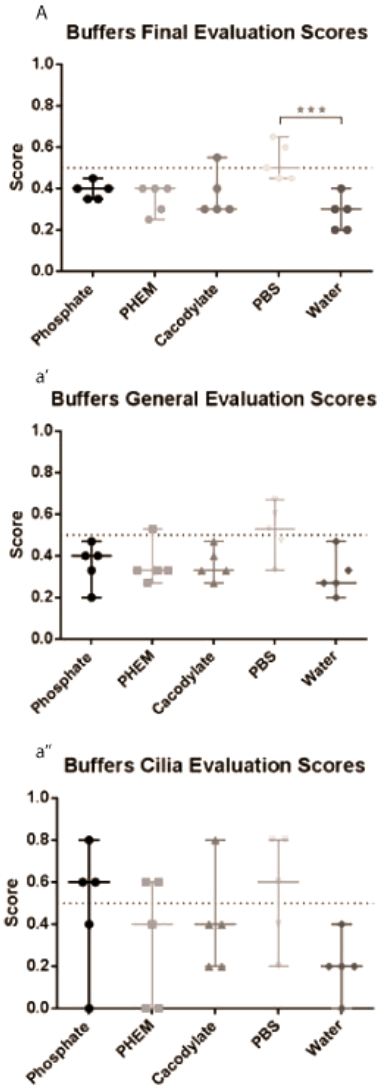

Fig. 1: Graphic Representation of the scores obtained for the different buffers used in chemical fixation.

As shown in Figure 1 (A), there is a significant statistical difference in the final score between water and PBS showing that not buffering the fixative solution can decrease the quality of ultrastructural preservation.

When comparing the data from the general evaluation scores Figure 1 (a') an odd result comes through the observation of the data. There is no major difference between water and the other buffers. It is believed that unbuffered fixative solutions produce inferior results than buffered fixatives (Dawes, 1971; Crang, R., Klomparens, K., 1988). However, the results obtained don't depict that inferiority. It can be that the evaluation table is not sensitive enough for the score obtained to reveal the inferiority exhibited in the panel pictures of this condition. If less stringent criteria were used it could be that the buffers would score higher which would comparatively decrease the evaluation of the water. Also, it might be that the magnification of the pictures of certain areas is not high enough for the artifacts to come across, since the areas evaluated at higher magnification (12k), the mitochondria and cilia, are the areas with very low scores for water.

Cilia preservation in the transition zone of the olfactory neurons in the antenna is similar between buffers but lower for unbuffered fixatives

If we analyze the data from the cilia evaluation scores Figure 1 (a'') regarding the comparison between buffers we can observe that the water evaluation score is much lower than the others, consistent with the idea that some organelles are more sensitive to buffering (Hayat, 2000). Cilia are highly specialized and have different membrane components than other organelles. This might lead to a different membrane diffusion of the fixative to the interior of the cilia, requiring more buffering function than other cellular components to protect the structure from the harmful fixative action. Another interesting finding is that Phosphate, Cacodylate and PBS are able to achieve the same high score, very close to the maximum possible and PHEM achieves a little less than them.

Although there are no significant differences between buffers, PBS shows better general ultrastructural preservation of the antenna

Looking at Figure 1 (A) and (a') it is visible that there is no significant statistical difference between buffers. However, PBS shows a slightly higher score for the final and general ultrastructural preservation. According to Weakley (1981), formaldehyde and glutaraldehyde are small molecules

that can easily penetrate the tissues, giving the buffer less importance in tissue preservation than for example with osmium tetroxide fixative. It is therefore normal that the difference in the ultrastructural preservation obtained with different buffers is not ample. This is also supported with the observations of Maunsbach (1966) and Busson-Mabillot (1971) were no differences were found with differently buffered glutaraldehyde fixatives, so long as the buffers have approximately the same osmolarity. Osmolarity is a key element to reduce cell shrinkage or swelling. The gradient should be kept as small as possible between intra- and extracellular space otherwise water will flow in one direction or the other. Another reason for the small differences observed between buffers can be related to the fact that the proteins in the tissue help to keep pH in a narrow range, and therefore the tissue functions as a buffer itself (Claude, 1962; Glauert, 1975; Crang, R., Klomparens, K., 1988). This could mean that the main importance of a buffer in a tissue is not its buffering capacity but actually the capacity of improving the fixative action. This can be achieved for example if the buffer gives specific ions to the fixative solution which can help the penetration of the fixative into the tissue or help to maintain slightly

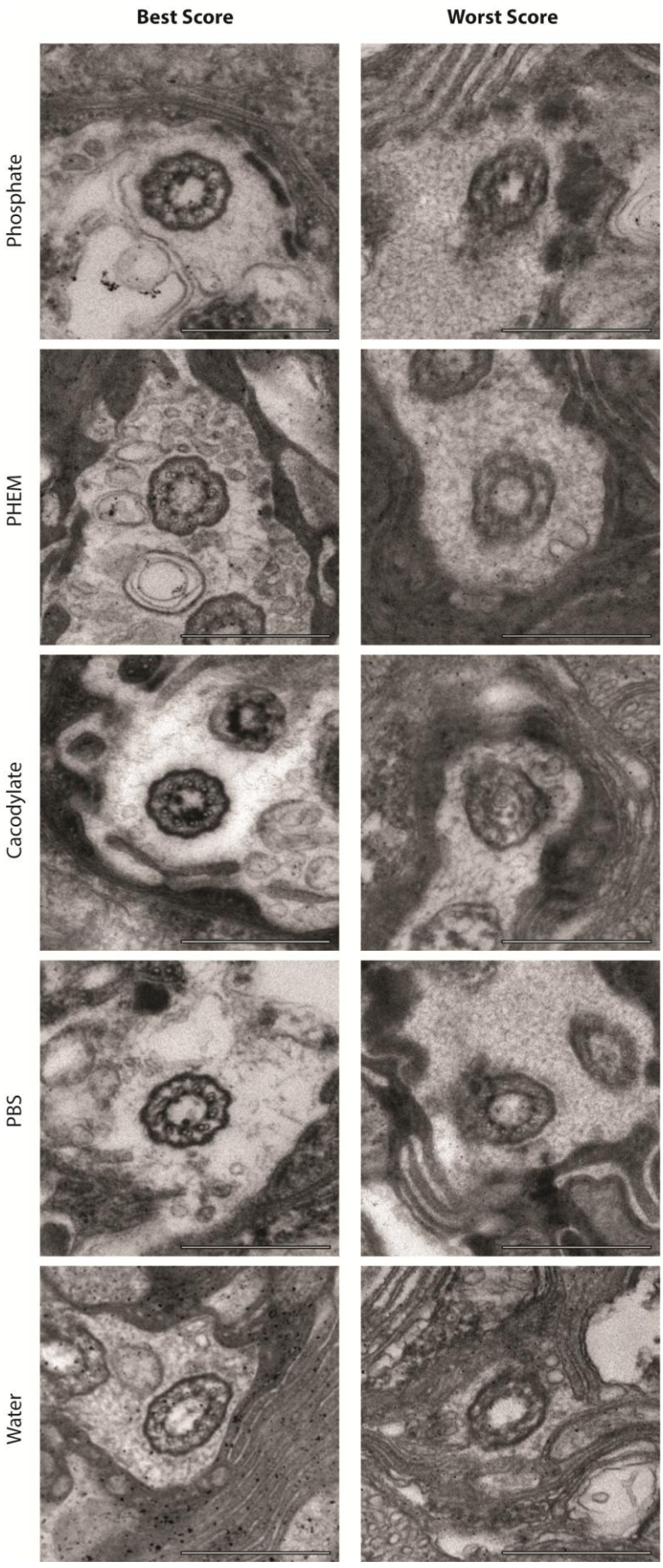

Fig. 2: Pictures representing the best and worst cilia scores for all the different buffers analyzed in the chemical fixation protocol. Scale bars represent 500 nm.

higher osmolarity in the fixative solution. It might be that the ions that PBS buffer contain do this function and help to slightly improve the general ultrastructural preservation in these samples.

Extraction of cytoplasmatic components can help to improve the visualization of certain cellular components.

If we analyze the pictures from Figure 2 we can easily correlate the better scores achieved by these buffers with the level of extraction they cause in the sample. It seems that the more extracted the cilia matrix is, the easier it is to visualize the microtubules and the y-linkers. The latter structures were never visible with PHEM, since this buffer has a much lower extractive action, preserving the cilia cytoplasm making it denser and conferring less contrast to the other structures. This is also a very interesting finding since it goes against the belief that extraction is always harmful. Obviously with extraction comes information loss (Crang, R., Klomparens, K., 1988) but if the structure that we want to analyze is actually not altered then the greater relative contrast gained with the extraction of cytoplasmatic matrix can be very helpful, as portrayed in this situation.

Fast Freeze Substitution wields good ultrastructural preservation of the antenna

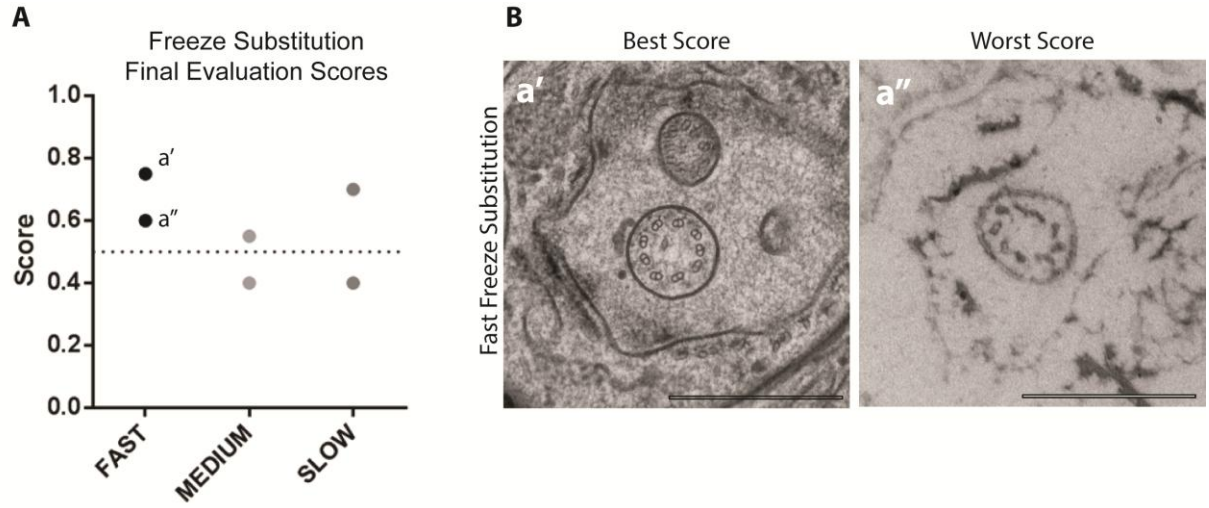

Fig. 3: A) Graphic representation of the final evaluation scores obtained for cryo fixation. B) Cilia pictures of the best and worst score obtained with the fast freeze substitution protocol. Scale bars represent 500 nm.

This study was more focused on chemical fixation since it is the routine technique in the majority of the electron microscopy laboratories in Portugal. However, a brief study of cryo fixation was also one of the goals, given that this technique has gained importance over the last years.

Before discussing the results, it is important to emphasize that the conclusions are based on a very low number of samples, so it is very hard to extrapolate our results. Despite the low sample number, some interesting results were observed when comparing freeze substitution protocols of different lengths.

The Fast Freeze substitution protocol showed good results for the ultrastructural preservation of the sample as shown in Figure 3. This result was expected since there are protocols for fast freeze substitution in *Dm* (although for different organs than the antenna) published showing good results

(McDonald, 2012; 2014). This is a very encouraging result since less time doing freeze substitution implies having results much faster, having less equipment time being used and spending less technician time and therefore decreasing the overall cost of the technique.

Besides being a very time consuming and expensive protocol, cryo fixing samples achieves a very low sample survival rate. This means that medium and low freeze substitution time are not necessarily bad for the overall quality of the samples but only that the samples analyzed were not preserved due to ice damage. The truth for this technique seems to be that the majority of the samples are badly preserved but the ones that are correctly cryo fixed show a very good ultrastructural preservation, even better than chemical fixation (Figure 4), as has already been described in the literature.

To identify the real morphology of a cell component it is necessary to combine chemical and cryo-fixation techniques

Analyzing the pictures from both techniques (Figure 4) we can see that both can achieve good structural preservation if the protocols are adapted to preserve the structure of interest. This means that both techniques have their own advantages. In the case of chemical fixation, although we have some artifacts like the membrane contour having a flower shape pattern, we can see the y-linker structures that are not visible by cryo fixation. However, cryo fixation preserves much better the cilia membrane maintaining it round. Therefore, what we can conclude from this result is that the best way of obtaining reliable morphological, biochemical and physiological information is to combine several techniques and to compare the tissue or cellular ultrastructure of specimens fixed with the different techniques (Crang, R., Klomparens, K., 1988).

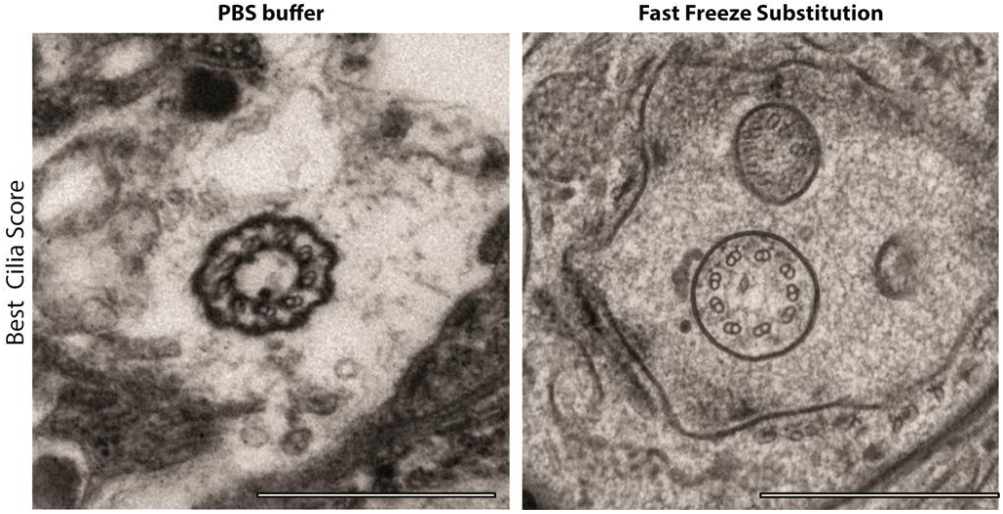

Fig. 4: Representative picture of the best score for the different fixation techniques. On the left column is represented chemical fixation using PBS buffer and on the right column is represented cryo fixation using the fast freeze substitution protocol. Scale bars represent 500 nm

Conclusion

This study gives further insights on the contribution of each method of fixation (chemical and cryo) to preserve the ciliary structure in the third segment of the *Dm* antenna and what kind of artifacts arise from each preservation technique. Also, having an evaluation table that summarizes the desirable characteristics of a sample processed for electron microscopy observation helps to understand what to look for in the sample when looking for artifacts specific for each technique. It is also helpful to have a tool that helps to compare different samples and even different techniques not only a qualitative way but also in a quantitative way. As shown here, a huge amount of information can be extracted when the samples are analyzed this way and this evaluation table has shown to be a valuable tool not only for experts in electron microscopy but also for people without any background in the area that might want to start doing some work in this field.

As a final remark, this study raises the question of what is the true action of a buffer in a fixative solution, since until now not much is known and discussed regarding this subject. It would be of great importance if in the future someone would address this question with a detailed chemical analysis. Also, this study allowed me to assess the chemical fixation protocol that preserves better the structures my research group is interested in studying. At the same time it gave me the opportunity to work with a new technique (cryo fixation) from which I was able to apply a protocol that provides good ultrastructural preservation. Moreover, cryo fixation might be useful to investigate further the ultrastructure of cilia in the antenna of *Dm* and maybe incorporate it in other studies such as immune detection of ciliary proteins in the transition zone.

Last but not the least, from this study arises a new tool, an evaluation table for judging ultrastructure. This table will be very helpful since it can be adapted to whatever sample or criteria being analyzed and it can be specified accordingly to a structure of interest. This can be applied not only for my routine in the laboratory to assess the quality of the samples I will work with, but also for everyone that is interested in standardizing new protocols, comparing techniques or even for routine quality control in electron microscopy facilities.

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