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LETTER TO THE EDITOR

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Comment on "Quality of extracellular vesicle images by transmission electron microscopy is operator and protocol dependent"

Dear Editor,

I read with interest the article by Rikkert and colleagues "Quality of extracellular vesicle images by transmission electron microscopy is operator and protocol dependent" [1]. In this article, two approaches for imaging extracellular vesicles (EV) by negative staining electron microscopy (EM) are compared. One approach consists in recording images at locations selected by the operator, while in the second approach, images are recorded at predefined locations on an EM grid, that is, at random locations not selected by the operator. The Authors conclude that images recorded at predefined locations reflect the overall quality of EV samples, while the operator selection approach is less suitable to evaluate the sample quality and leads to results influenced by operator bias.

I wish to express my disagreement with this overall conclusion. I consider that, in the case of heterogeneous samples like EV, analyzed by negative staining EM, it is preferable to record images at operator selected positions, unless and until optimized preparation protocols are applied. In my opinion, the Authors make a confusion between the sample quality, defined in the article as the content and purity in EV, on the one hand, and the quality of the preparation protocol, on the other hand.

The aim of an EM study is to provide a faithful description of a sample; in the case of EV samples, this means describing the morphology and size distribution of EV, the presence or absence of non vesicular particles and aggregates, and determining the relative amount of the various components. Imaging a sample by negative staining EM involves three consecutive steps, namely (1) adsorption of the material contained in a sample on a carbon support film, (2) staining and air-drying, and (3) EM imaging per se [2,3]. Step (3) is a quasi ideal operation, in the sense that the recorded image is an exact representation, magnified, of the material present locally on the grid (at 2-nm resolution for negative staining). On the other hand, steps (1) and (2), which together constitute the preparation protocol, are complex and poorly controlled processes [2–4]. Ideally, the material deposited on the carbon film should reflect faithfully the sample content, and the material and stain deposit should be homogeneously distributed over the entire EM grid. With such an ideal preparation protocol, equivalent results would be obtained by imaging a sample at predefined locations and at operator selected locations.

However, anyone with some experience in EM of negatively stained samples is aware that the actual situation is different. Most often, the amounts of adsorbed material and stain deposit vary across the grid. This reflects the variability in the local adhesion properties of the support film and is a sign of preparation protocols of poor quality. Results obtained by three out of the four protocols selected by Rikkert and colleagues correspond to this situation, as clearly illustrated in Figures 5–7 of [1]. In this case, images recorded at predefined locations are likely to be impacted, if not dominated, by flaws from the preparation protocol, and are thus expected to provide an incorrect representation of the sample itself. This is the reason why, in common practice, the operator scans the EM grid at low magnification and searches for areas which present a larger amount of adsorbed material and/or a more homogeneous distribution of material and stain. It is logical and legitimate that the operator selects these areas and not areas of lesser quality in terms of material spreading and staining. In doing so, the operator applies a conscious bias, based on his/her experience and knowledge from previously published litterature.

In conclusion, current efforts towards the development of optimized preparation protocols for EV imaging by negative staining EM, as presented in Rikkert and colleagues' article, are highly valuable. This is a challenging task, given the heterogeneity and diversity of EV samples, e.g. from body fluids or cell conditioned media [5,6], and the large variety in carbon film adhesion properties [4].

Finally, in the context of this article, a major advantage of cryo-EM over negative staining deserves to be emphasized. Indeed, in cryo-EM, objects are (in general) not directly adsorbed on a support film, but are contained within frozen nanodroplets suspended over a perforated film [7,8]. The absence of adsorption issue correlates with

the highly homogeneous distribution of objects, which characterizes cryo-EM grids [9]. Therefore, in the case of cryo-EM, it is forseeable that imaging EV samples at predefined positions, say, in a fully automated manner or with minimal intervention from the operator, will become a routine approach.

Disclosure statement

No potential conflict of interest was reported by the author.

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