Minireview

Live-Cell Structural Biology to Solve Biological Mechanisms: The Case of the Exocyst

Ibai Irastorza-Azcarate[1, 4]

Daniel Castaño-Díez[2]

Damien P. Devos[1]

Oriol Gallego[3, *]

oriol.gallego@upf.edu

[1]Laboratory of Evolutionary Innovations, Centro Andaluz de Biología del Desarrollo, CSIC, Universidad Pablo de Olavide, Carretera de Utrera, Km. 1, 41013 Seville, Spain

[2]BioEM Lab, Center for Cellular Imaging and Nanoanalytics, Biozentrum University of Basel, Basel, Switzerland

[3]Department of Experimental and Health Sciences (DCEXS), Pompeu Fabra University (UPF), 08003 Barcelona, Spain

*Corresponding author

[4]Present address: Berlin Institute for Medical Systems Biology, Max Delbrück Center for Molecular Medicine, 10115 Berlin, Germany

Historically, structural biology has been largely centered on *in vitro* approaches as the dominant technique to obtain indispensable high-resolution data. *In situ* structural biology is now poised to contribute with high-precision observations in a near-physiological context. Mass spectrometry, electron tomography, and fluorescence microscopy are opening up new opportunities for structural analysis, including the study of the protein machinery in living cells. The complementarity between studies is increasingly used to reveal biologically significant observations. Here we compare two complementary studies addressing the mechanisms of vesicle tethering with *in vitro* and *in situ* approaches. Cryoelectron microscopy and live-cell imaging assisted by anchoring platforms team up to explore elusive mechanisms of exocytosis, showing directions of future research.

Irastorza-Azcarate et al. summarize recent cryo-EM and live-cell imaging studies on the exocyst, a protein complex involved in tethering secretory vesicles to the plasma membrane in exocytosis. The comparative analysis demonstrates the power of integrating *in vitro* and *in situ* approaches to unravel the structure and mechanism of macromolecular complexes.

Main Text

Structural information is key to understand the function of the cellular machinery. Ideally, experimental observations devoted to resolve biological mechanisms should achieve resolutions at atomic scale and be realized in conditions close to the physiological environment. However, until recently, *in vitro* approaches have occupied the front seat of structural biology. A compromise between resolution and biological significance needs to be found (Figure 1). X-ray crystallography has been for many decades the ultimate method to provide structures at atomic resolution. To form the crystals, proteins and their complexes are purified and taken away from their physiological context, while resolved structures are still snapshots of flexible entities. Therefore, crystallography biases toward more stable conformations that can be homogeneously isolated, potentially neglecting more dynamic physiological states. NMR allows determining atomic structures in solution, but this method is limited to proteins of small size. Recently, cryoelectron microscopy (cryo-EM) circumvented some of the burden related to the need to obtain crystals with competitive resolution. However, protein complexes still need to be purified and isolated from their physiological environment in the cell. Even if cryo-EM can resolve heterogeneous populations of conformations, in practice, the resources required to determine low abundant species limit the space of conformations that can be effectively explored and the characterization of structural dynamics. *In vitro* methods cannot directly assess the functionality of the resolved sample. To corroborate that resolved structures correspond to physiological conformations, *in vitro* approaches require secondary *in vivo* assays that cannot completely eliminate uncertainty. Although *in vitro* approaches provide necessary high-resolution structures, the gap between resolution and physiological relevance persists.

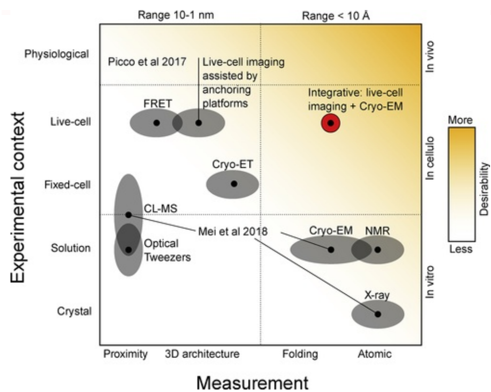

Figure 1 Research Space Overview in Structural Biology

Area of research embraced by some of the main methods used to study molecular structures in biology (brown). Studies on the 3D organization of the exocyst using *in vitro* and *in situ* approaches are highlighted together with the methods that they used. Integration of these studies achieves a better resolution of structural details in a more relevant context (red).

In situ (also known as *in cellulo*) structural biology, the study of structural properties of biomolecules in their cellular context, tries to fill the gap toward the comprehension of biological mechanisms at atomistic scale and in physiological environments. Multiple approaches have been developed, such as protein crosslinking mass spectrometry (CL-MS) and cryoelectron tomography (cryo-ET). CL-MS is a powerful technique that uses chemical reactions to map the vicinity between peptides. This has been very useful to identify specific sites of protein-protein interactions or to study conformational changes occurring in the cell (O'Reilly and Rappsilber, 2018; Wang et al., 2017). Cryo-ET employs electron microscopy to obtain 3D tomograms of frozen biological samples. In a process related to single-particle analysis, subtomogram averaging combines the *in situ* imaging of multiple copies of the targeted complex to determine its structure at the angstrom scale (Hutchings and Zanetti, 2018). However, inherent limitations of chemical crosslinking and cryopreservation continue preventing these methods to resolve the cellular machinery in a functionally dynamic environment.

Light-based approaches offer unique opportunities to investigate the cellular machinery with relatively few resources. Structural analysis by optical tweezers employs tightly focused laser beams to trap reconstituted molecular assemblies that are attached to synthetic beads. This method is capable of measuring, at the single molecule level, conformational changes in the order of low-nanometer and forces in the piconewton scale with a submillisecond time resolution (Choudhary et al., 2019; Ritchie and Woodside, 2015). Fluorescence microscopy can also be implemented to perform more physiological live-cell imaging and to pinpoint molecular identities with fluorescent tags. As a downside, fluorescence microscopy shows insufficient resolution to investigate the details of biochemical entities *in vivo*. While super-resolution microscopy has resolved very large multi-component assemblies with a 10–20-nm resolution (Huang et al., 2016; Loschberger et al., 2012; Mund et al., 2018), this is not enough to resolve the vast majority of molecular assemblies. Alternatively, when two fluorophores cannot be spatially resolved, fluorescence microscopy can use optical filters to spectrally resolve them. Förster resonance energy transfer is capable to measure distances between spectrally different fluorophores in the range of <10 nm (Hellenkamp et al., 2018; Sakon and Weninger, 2010). Localization microscopy identifies the centroid position of spectrally different fluorophores in the image to measure their separation with a precision in the order of few nm (Churchman et al., 2005, 2006). Both methods have been used to map the subunit organization of the kinetochore during metaphase (Aravamudhan et al., 2014; Wan et al., 2009). However, to integrate distance measurements between labeled proteins, it is necessary to have spatial references inside the cell where the measurements are taken. These approaches could not be generally applied to reconstruct the 3D architecture of protein complexes *de novo*.

Recently, a fluorescence microscopy method was developed to determine *de novo* the 3D architecture of protein assemblies directly in living cells. This approach is based in the Protein interactions from Imaging Complexes after Translocation (PICT) assay that was originally developed to study protein-protein interactions by live-cell imaging (Gallego et al., 2013). PICT employs the rapamycin-induced dimerization (Chen et al., 1995) to recruit specific protein complexes labeled with GFP to intracellular anchoring platforms tagged to RFP (Gallego et al., 2013; Torreira et al., 2017). To reconstruct the architecture of protein complexes, anchoring platforms have been modified to form immobile flat surfaces, of smaller size than the diffraction limit and that can be reproducibly assembled through the population of imaged cells. Anchoring the GFP-tagged protein complex boosts the capabilities of live-cell imaging to measure the separation between RFP and GFP with an approach derived from localization microscopy. The measurement of the RFP-to-GFP distance is repeated in yeast strains where GFP is fused to different positions of the protein complex while RFP is always attached to the anchoring platform. Thus, the RFP tag provides a spatial reference to integrate the set of distances and to map in the 3D space each of the subunit termini that has been fluorescently labeled with GFP. Although the reported structural details are far from the capabilities of cryo-ET or *in vitro* techniques, live-cell imaging assisted by anchoring platforms provides a unique opportunity to monitor simultaneously the 3D architecture of a complex and its function (Picco et al., 2017).

Here we perform a comparative analysis between recent *in vitro* and *in situ* structural biology studies that have explored the mechanism of exocytosis. The integration of different source of information is a promising technique to fill the gap between resolution and biological significance. The combination of biochemical assays upon protein depletion (Heider et al., 2015), structure reconstruction by cryo-EM and CL-MS (Mei et al., 2018), and live-cell imaging assisted by anchoring platforms (Picco et al., 2017), allows us to model the mechanism of vesicle tethering.

Exocytosis is a vesicle-trafficking pathway conserved in all eukaryotes that is responsible for delivering biomolecules to the cell surface and extracellular media (Rothman, 1994). Exocytosis is fundamental for cell growth and cell polarity, and as such it also plays a critical role in a number of human pathologies including cancer and neurological disorders (Dixon-Salazar et al., 2012; Heider and Munson, 2012; Martin-Urdiroz et al., 2016; Noor and Zahid, 2017; Zeng et al., 2017). Multiple protein complexes, regulatory proteins, and membranes of different chemical composition form transient assemblies that control this pathway with elusive modes of action. Understanding the mechanism of exocytosis requires structural knowledge of the proteins involved, how they assemble and the conformational dynamics that drive molecular functions. However, because this machinery could not be functionally reconstituted *in vitro*, the insight derived from its structural characterization has been limited. Up to now, exocytosis remains a largely unknown process at the mechanistic level.

The exocyst is a conserved hetero-octameric protein complex responsible for tethering secretory vesicles to the plasma membrane during exocytosis. Early EM imaging of the mammalian exocyst suggested that the exocyst is a highly flexible complex that might adopt different conformations (Hsu et al., 1998). The molecular details involved in exocyst activity are not clear, but in recent years the structural characterization of this protein complex has advanced enormously (Lepore et al., 2018). About 30% of the exocyst has been solved by X-ray crystallography of truncated subunits (Baek et al., 2010; Chen et al., 2017; Dong et al., 2005; Fukai et al., 2003; Hamburger et al., 2006; Jin et al., 2005; Sivaram et al., 2006; Wu et al., 2005; Yamashita et al., 2010). These structures suggest that exocyst subunits are largely formed by helical bundles repeats (Croteau et al., 2009; Munson and Novick, 2006).

The structural characterization of the intact exocyst was first challenged *in vitro* with the analysis of its shape and hierarchy of interactions among the eight subunits. Biochemical experiments analyzed the stability of the complex upon depletion of individual subunits (Heider et al., 2015; Katoh et al., 2015). The exocyst was found to be organized in two modules of four subunits each. The subunits Sec3, Sec5, Sec6, and Sec8 locate in tetramer 1, while subunits Sec10, Sec15, Exo70, and Exo84 form tetramer 2 (Figure 2A). In addition, quick-freeze deep-etch and negative-stain electron microscopy showed that the exocyst subunits present an elongated rod shape compatible with a helical bundle fold (Heider et al., 2015; Hsu et al., 1998).

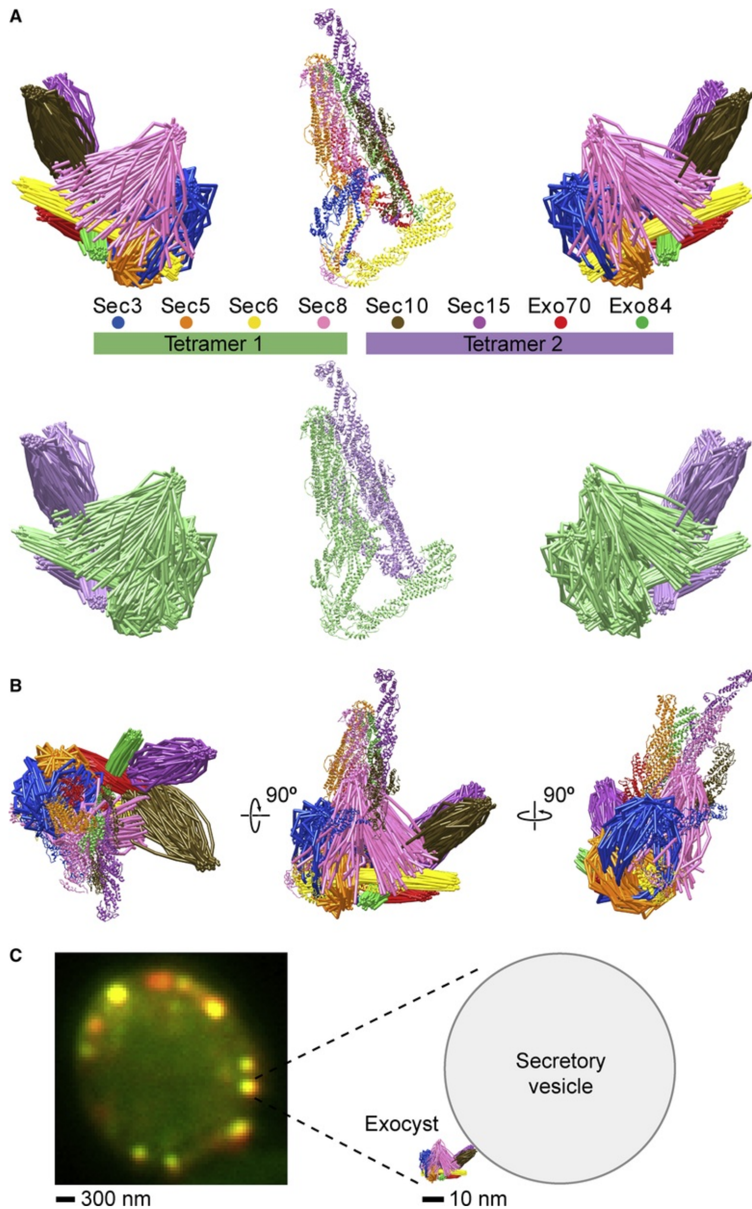

Figure 2 The Exocyst Determined by *In Vitro* and Live-Cell Structural Biology

(A) The two mirror images of the exocyst architecture obtained by live-cell imaging assisted by anchoring platforms (Picco et al., 2017) are shown (left and right). Each component of the complex is represented as a flexible string, and all conformations compatible with distance measurements are superposed. The near-atomic structure (center) obtained *in vitro* (Mei et al., 2018) identifies the correct mirror image as the one to the right. Complexes are color coded by subunits (top) and tetramers (bottom).

(B) Superposition of the exocyst determined *in vitro* and in living cells. Sec6 (yellow) and Sec8 (pink) were used for the alignment.

(C) Fluorescence image of an engineered yeast cell (left) with membrane-associated anchoring platforms labeled with RFP. The entire exocyst, including Sec5-GFP, is anchored to the platforms (RFP-GFP colocalization). Integration of distance measurements obtained from live-cell imaging allowed the reconstruction of the architecture of the exocyst when bound to a secretory vesicle. The vesicle is shown to scale with the complex (right).

Last year, a near-atomic model of the purified exocyst was reported by cryo-EM. CL-MS of the purified complex was used to position adjacent subunits in the cryo-EM data. Atomic-resolution structures derived from X-ray crystallography and comparative modeling, were fitted according to the cryo-EM density to generate a more detailed model (Mei et al., 2018). The exocyst presents a hollow structure with an elongated shape of 318 Å in length, where most of the protein subunits are packed contiguously. Even if it did not reach atomic resolution, the *in vitro* 3D structure of the exocyst captured well the tetrameric modules and further resolved the molecular details that mediate their binding (Figure 2A) (Mei et al., 2018). Exocyst subunits fold in helical bundles, which results in their elongated conformation. In addition, they also present a long coiled-coil N-terminal stretch, the conserved Core of Exocyst (CorEx) motif, which mediates the interactions necessary to build the tetramers. Likely because of its flexibility, the Sec3 N-terminal half could not be located within the structure. Sec10 and Sec15 were resolved as adjacent rod-shaped proteins in a conformation compatible with their function in vesicle binding as a dimer (Mei et al., 2018). However, no vesicle tethering assay has been established to assess the functional state of the purified exocyst and the binding to the vesicle could not be reconstituted *in vitro*.

The structural analysis of the exocyst has also been addressed by live-cell imaging assisted by anchoring platforms (i.e., PICT) followed by 3D architecture determination by integrative modeling (Picco et al., 2017). Fluorescent tags labeled the anchoring platform and one of the exocyst subunits at a time (N- or C-terminally). Since exocyst assembly and function is essential for yeast growth (Wiederkehr et al., 2004), distance measurements were performed exclusively for GFP fusions in cells with normal growth rate (Picco et al., 2017). Fluorescence recovery after photo bleaching experiments demonstrated that recruited exocyst is fully assembled. Secretory vesicle binding supported functionality of the exocyst when it is recruited to the anchoring platform (Picco et al., 2017). Thus, functional assays simultaneously performed to the structural characterization demonstrated that the *in situ* reconstructed exocyst is functional.

Anchoring the exocyst provided an optimal cellular context to measure the separation between fluorescent tags. The distance to the anchoring platform could be measured for all fluorophores labeling the subunits with a precision of 5–2 nm. These distances were integrated with available structural information of the subunits to reconstruct the 3D architecture of the complex. Each component of the exocyst was represented as a flexible string. Note that the precision of the distance measurements tolerated that modeled strings adopt different configurations as long as they were compatible with the data obtained by live-cell imaging. Nonetheless, live-cell imaging assisted by anchoring platforms positioned each of the exocyst subunits in a well-defined space within the architecture (Figure 2A) (Picco et al., 2017).

Although this reconstruction of the exocyst provided two mirror-image solutions, both of which fulfill the distances restraints in exactly the same way, the structure of the purified complex allowed to resolve the mirror-image ambiguity (Figure 2A). This grants us with a unique opportunity to explore the complementarity between *in vitro* and *in situ* characterization of this fundamental piece of the exocytic machinery.

In the live-cell reconstruction, the two tetrameric modules are clearly defined in agreement with the biochemical data (Figure 2A). Exocyst subunits present a rod-like shape, with most of their N termini forming a tightly interconnected mesh at the core of the complex, while the other termini project toward the periphery (Figure 2B). The position of Sec3 N terminus and Exo70 C terminus is compatible with their simultaneous binding to the plasma membrane. The exocyst architecture determined in living cells corresponds to a secretory vesicle-bound form of the complex, as the anchored exocyst preserves its ability to bind secretory vesicles. Thus, the secretory vesicle could be mapped within the architecture of the exocyst (Figure 2C). Sec10 is contiguous to Sec15, and both proteins extend, parallel to each other, from the core of the exocyst to the surface of the secretory vesicle (Picco et al., 2017).

Despite the good agreement between live-cell imaging and the cryo-EM characterization of the exocyst, remarkable differences are also observed. Using the same representation of the exocyst subunits, we could estimate an average root-mean-square deviation (RMSD) of 88.9 Å between the isolated and the *in situ* vesicle-bound exocyst. Individually, both tetrameric modules superpose well with their counterpart, but the *in vitro* and the *in situ* exocysts differ in the relative position of the two tetramers. Whereas the exocyst bound to the vesicle adopts the conformation of an open hand, the isolated exocyst presents a “closed” conformation with the two tetrameric modules being more compacted (Figures 2B and 3A). Taking tetramer 1 as a reference, tetramer 2 appears to be displaced by a rotation of 69°. Rotating tetramer 2 accordingly reduces the RMSD by 31% (Figure 3B). Indeed, tetramer 2 (including Sec10 and Sec15), but not tetramer 1, has been shown to bind Sec4, a Rab GTPase present on the surface of secretory vesicles (Guo et al., 1999). Overall, these data suggest that the exocyst function involves a structural rearrangement between the two tetrameric modules from a “close” conformation (isolated exocyst) to an “open” conformation (exocyst bound to the vesicle). The presence of a large cavity at the interface between the two tetrameric modules in the cryo-EM structure is compatible with such a rotational movement (Figure 4).

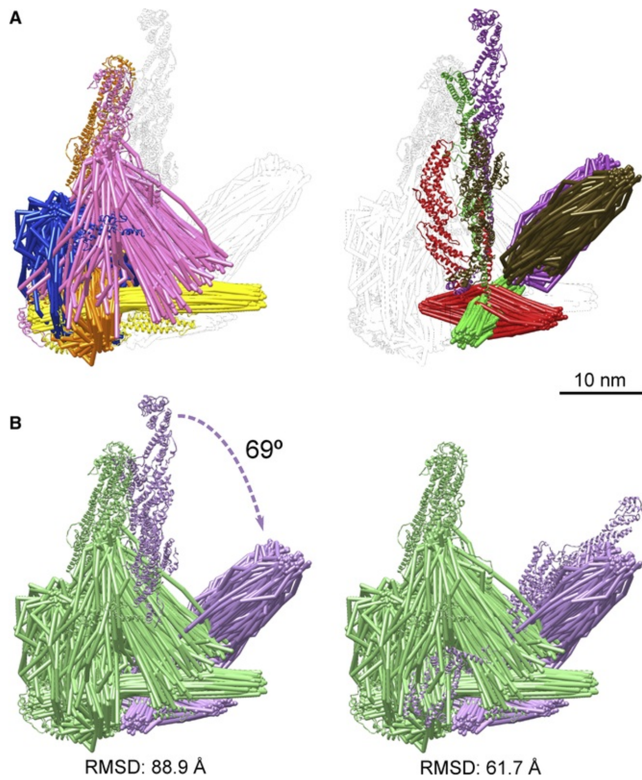

Figure 3 Rotation of Tetramer 2 Accounts for the Main Differences between Isolated and Vesicle-Bound Exocyst

(A) Superposition of the isolated exocyst and the vesicle-bound exocyst. Complexes are color coded by subunits as in Figure 2. Transparency highlights subunits in tetramer 1 (left) and tetramer 2 (right).

(B) Superposition of the isolated exocyst and the vesicle-bound exocyst. Complexes are color coded by tetramers as in Figure 2. Arrow indicates the rotation of tetramer 2. RMSD values are the average RMSD calculated from the superposition of both structures, before (left) and after (right) rotation by 69° of tetramer 2 of the isolated exocyst. To calculate the RMSD, the exocyst subunits were represented as in Picco et al. (2017).

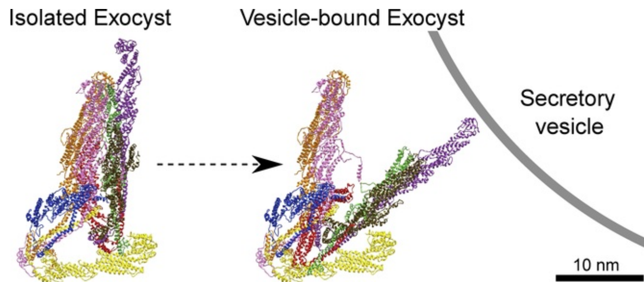

Figure 4 Mechanistic Model for the Activation of the Exocyst

Possible model for the structural rearrangement of the exocyst upon vesicle binding. Complexes are color coded by subunits as in Figure 2.

Differences also arise when comparing the two reconstructions in more detail. While most of the subunits trace a similar conformation in both reconstructions, Sec10 presents some variability. In the cryo-EM model, Sec10 adopts a "U" shape with both N and C termini at the periphery of the exocyst, close to the Sec15 C terminus. Instead, in the *in situ* reconstruction, Sec10 adopts a rod-like shape with only the N terminus located at the periphery of the complex. A hinge between the N-terminal CorEx motif and the helical bundle is compatible with a rotational movement of the Sec10 C terminus, which could explain the two conformations observed. However , although yeast-two

hybrid supports the architecture observed by live-cell imaging, insufficient experimental data are available to resolve this apparent discrepancy. In any case, this analysis illustrates well the need for integrative *in vitro* and *in situ* approaches to reconstruct the structure of protein complexes with higher structural detail and to capture conformational changes that are relevant for molecular functions.

This comparative analysis shows that the development of intracellular nanotools allow quantitative live-cell imaging to position proteins in the 3D architecture of multi-subunit complexes. Despite being less invasive than cell lysis and purification, controls have to be considered to ensure that the employment of intracellular nanotools is innocuous for the complex. Eventually, the use of anchoring platforms could be applied to resolve, *de novo*, any cellular assembly that can be efficiently tagged and anchored. For example, this method was also used to determine the molecular organization of the conserved oligomeric Golgi complex, a multi-subunit tethering complex involved in the Golgi retrograde transport (Picco et al., 2017).

Differences observed when the results derived from the *in vitro* isolated and the *in situ* vesicle-bound conformations of the exocyst are superposed might inspire novel mechanistic insight. As the capabilities to bind secretory vesicles differ between the isolated and the cellular exocysts, we suggest that differences in their conformation might be related to the function of the complex. Recently, new live-cell approaches have added to the characterization of the molecular basis of the exocyst function. Total internal reflection fluorescence microscopy and fluorescence cross-correlation spectroscopy showed that the mammalian exocyst is a two-part complex composed of the corresponding tetrameric modules. Tetramer 1 and tetramer 2, which associate in a dynamic equilibrium, arrive at the plasma membrane with a short, yet functionally relevant, ~80-ms delay (Ahmed et al., 2018). Thus, full complex assembly, vesicle binding, and induced conformational changes form a sequence of events that somehow control exocyst activity, and which in turn determine the process of exocytosis. Such conformational dynamics and the mechanism of regulation will need to be tested experimentally. Nevertheless, the analysis of the exocytic machinery illustrates well how integration of *in situ* structural biology may provide fundamental experimental data to interrogate biological mechanisms beyond the limits of *in vitro* approaches (Figure 1).

Only the development of innovative techniques to provide high-resolution structural details with minimal manipulation of the sample will allow understanding cellular mechanisms comprehensively. Nowadays, *in vitro*-derived atomic structures remain indispensable in this research, despite the fact that cryo-ET seems poised to provide high-resolution data *in situ* in the near future (it has already provided structures with a resolution of <12 Å in the cellular context [Delarue et al., 2018]). Several long-sought instrumental advances, such as focused ion beam milling (Schaffer et al., 2017) and correlative light-electron microscopy (Wang et al., 2012), have gradually managed to overcome cryo-ET technical difficulties to converge into an effective *in situ* visualization tool. These, and yet to come improvements (i.e., deep learning-driven alignment, classification, and segmentation) surely will multiply the cryo-ET applications to resolve (near-)atomic structures in the cell. However, cryo-ET is intrinsically limited to resolve “frozen” snapshots of the cellular machinery, limiting the insight of the dynamics. In addition, cryo-ET might not be able to resolve molecular identities within a complex, unless it reaches high resolution, a goal that is not attainable for every biological sample. Thus, structural biology field remains in the search for complementary methods. Although, fluorescence microscopy offers unique opportunities to resolve molecular identities and to perform structural analysis in living cells, the capabilities of live-cell imaging assisted by innovative molecular nanotools need to be further explored. In this scenario, it is clear that only the combination of *in vitro* and *in situ* approaches will achieve a detailed understanding of the cellular machinery (Figure 4). The complementarity between these techniques should be investigated by integrative methods and, ideally, include live-cell structural biology data.

Future developments will open up unexpected spaces of research closer to the ideal atomic-resolution real-time imaging of biomolecules in a totally physiological context. While this is not possible yet, emergence of innovative live-cell techniques will give rise to new integrative approaches in which the combination of *in vitro* and *in situ* methods can resolve biological mechanisms that could not be interrogated before (Kim et al., 2018). In the present analysis, the synergy of cryo-EM and live-cell imaging has overcome technical boundaries and has provided experimental ground to speculate about unexplored activation switches central to the mechanism of exocytosis.

Acknowledgments

We acknowledge Prof. C. Govaerts (ULB, BE) and Prof. M. Munson (UMMS, USA) for comments on the manuscript. I.I.-A. thanks the Helmholtz Association (11431). O.G. was supported by the “Unidad de Excelencia María de Maeztu,” funded by the MINECO (ref: MDM-2014-0370). D.P.D. and I.I.-A. were supported by a research grant from MINECO (ref: BFU-2016-7826-P) and by the “Unidad de Excelencia María de Maeztu,” funded by the MINECO (ref: MDM-2016-0687).

Author Contributions

I.I.-A., D.C.-D., D.P.D., and O.G. performed the analysis, discussed results, and wrote the manuscript.

Declaration of Interests

The authors declare no competing interests.

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Keywords: exocytosis; exocyst; molecular mechanisms in membrane biology; light microscopy; integrative structural biology; Cryo-ET; Cryo-EM

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