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Super-resolution microscopy to decipher multi-molecular assemblies

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Super-resolution fluorescence microscopy (SRM) is increasingly being applied as a complementary method to resolve the organization of large biomolecular assemblies. One of its main advantages is that it provides information on protein organization and identity simultaneously, within the native cellular milieu. It also extends the accessible range of structures up to the micrometer scale, offering complementary information relative to classical structural biology methods. Furthermore, SRM is capable of resolving the organization of some biomolecular assemblies not accessible to other methods. We highlight these advantages within the context of deciphering the structure of the centrosome and chromatin, and discuss how computational data post-processing has been adapted for SRM data. We also outline current limitations and potential approaches to overcome them.

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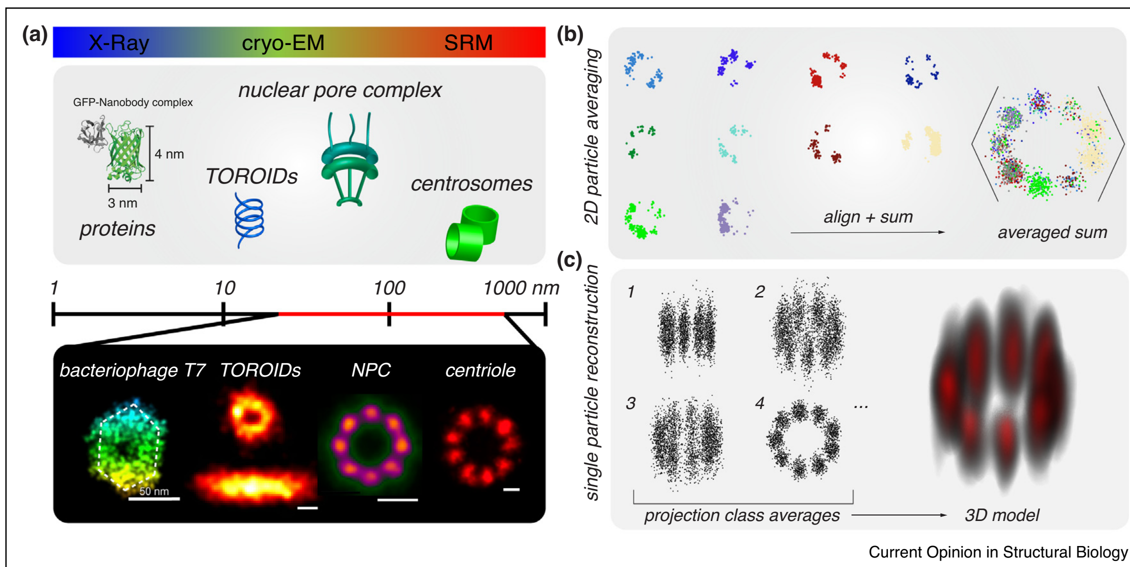
Introduction

A vast amount of knowledge of how proteins assemble and function has been revealed by classical structural biology techniques, such as X-ray crystallography, nuclear magnetic resonance spectroscopy (NMR) and electron microscopy (EM). Although these methods are well-adapted to study small macromolecular structures with resolutions approaching atomic scales, a gap remains for resolving larger macromolecular assemblies, up to the micrometer scale. Moreover, it can be challenging to identify the nature of individual components within a complex. By contrast, in fluorescence microscopy, genetic tags or specific antibodies selectively target only one molecule species at a time, providing simultaneous information about protein identity and context. Fluorescence super-resolution microscopy (SRM) may thus bridge the size gap, while providing complementary information, especially for the characterization of large multi-protein complexes *in vivo* (Figure 1a). A number of SRM techniques (Box 1) have matured over the past decade and are now well-established within the fluorescence microscopy repertoire of the cell biology community [1]. More recently, applications to structural biology have established the potential of SRM for revealing the organization of cellular compartments and machines (Figure 1a). As we will discuss below, SRM has been of particular interest for the study of large or amorphous complexes and for its compatibility with functional live-cell imaging.

However, for SRM to reach its full potential as an adequate tool in structural biology, it should address the following technical challenges: (i) collection of large ensembles of particles to capture sample heterogeneity, (ii) high-fidelity and high-density labelling, preferably site-specific at the scale of the constituent amino acids or base pairs, (iii) multi-color reconstruction, and (iv) sample preservation commensurate with the achieved resolution. The ideal SRM technology would combine all of these features to attain maximal resolution on multiple proteins and place them within three-dimensional volumes. Although no one method yet meets all of these criteria, individual improvements are continuously emerging which may in the future be powerfully combined.

In this review, we will highlight recent advances that helped to overcome some of these limitations and contributed to establishing SRM as a quantitative tool in structural biology. We will further discuss experimental approaches for studying large and rigid complexes, such as the centriole or the nuclear pore complex, versus those suitable for more amorphous structures, like chromatin. These classes of structures are fundamentally different, and in the case of an undetermined molecular complex, one first needs to consider its estimated organization, orientation and similarity with copies of itself. These aspects then help to determine the most suitable methods to reveal its structure.

Figure 1



Super-resolution microscopy allows to study multi-molecular complexes up to the micrometer scale, providing information complementary to X-ray crystallography and electron microscopy **(a)**. Notable structures (shown in a, lower panel from left to right), that were recently described using SRM approaches are the bacteriophage T7 [12], TORC1 inhibited domains (TOROIDs) found in yeast [19••], the mammalian nucleoporin gp210 within the nuclear pore complex (NPC) [7] as well as the distal appendage protein Cep164 within the centriole of mTECs [14••]. Scale bars = 100 nm. The crystal structure of the GFP–nanobody complex is shown for size comparison. A predominant limitation of SMLM is low labelling density and sample heterogeneity. To visualize this effect, we simulated a nine-fold symmetric structure **(b,c)**. One way to overcome this problem is particle averaging, where individual structures are aligned and averaged or simply overlaid (b). While individual imaged structures do not contain all structural information (e.g. a nine-fold symmetry, left side in b), this information is recovered after particle accumulation (b, right side). If enough particles where imaged at multiple orientations (c), those can be classified into projection averages and used for 3D single particle reconstruction.
Source: (a, lower panel) Reproduced from Refs. [7,12,14••,19••] with permission.

Super-resolution single particle averaging and reconstruction

The resolution in SRM is fundamentally limited by the requirement of densely labelled structures [2–4]. This is of particular importance in single molecule localization microscopy (SMLM), where the lateral localization precision routinely approaches ten nanometers. As an example, a 3D resolution of 10 nm requires a labelling density of 8×10^6 labels per μm^3. Considering the stochastic nature of SMLM, the typical size of an antibody ($\sim$10 nm) and the accessibility of existing antigens within the target, this becomes a tremendous challenge. In practice, the labelling is often too-sparse and heterogeneous, limiting the achievable resolution.

One way to overcome this problem is particle averaging, in a similar manner to that performed in EM. The simplicity and strength of SRM particle averaging is exemplified by a number of recently reported biological structures. One of the most beautiful examples is the nuclear pore complex (NPC), which also highlights the two requirements of 2D particle averaging: (1) large numbers of particles, (2) sharing the same orientation (Figure 1b). The NPC is a large multiprotein assembly with a diameter of about 120 nm integrated into the nuclear membrane, where it controls the transport of molecules between the nucleus and the cytoplasm [5]. A mammalian cell nucleus contains on average 3000 NPCs [6], all oriented within the nuclear envelope, thereby helping both requirements for 2D particle averaging to be fulfilled. The striking eight-fold symmetry of the nucleoporin gp210 as well as the central NPC channel could be resolved by 2D averaging and rotational alignment of several hundred reconstructed SMLM images [7] (Figure 1a). Later, the head-to-tail organization of the NPC y-complex was unravelled by deducing the radial position of seven subunits distributed along the complex [8]. A striking periodicity was also shown for the organization of actin and spectrin in neurons [9,10]. Here, the regular spacing between the actin rings could be extracted using a Fourier transformation of their 1D projection, revealing a spacing of 190 nm along the axon. While the NPC is relatively flat, with axial (transmembrane) dimensions of $\sim$50 nm [5], particle averaging has also been applied to larger 3D complexes such as viruses [11•,12,13] or centrioles [14••,15•,16,17•], spanning several hundred nanometers.

Extended complexes require the acquisition of larger particle numbers and the development of suitable filtering protocols to classify particles into groups with the

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