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Review

Challenges in quantitative single molecule localization microscopy

A. Shivanandan, H. Deschout, M. Scarselli [1], A. Radenovic *

Laboratory of Nanoscale Biology, Institute of Bioengineering, School of Engineering, EPFL, Lausanne, Switzerland

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ABSTRACT

Single molecule localization microscopy (SMLM), which can provide up to an order of magnitude improvement in spatial resolution over conventional fluorescence microscopy, has the potential to be a highly useful tool for quantitative biological experiments. It has already been used for this purpose in varied fields in biology, ranging from molecular biology to neuroscience. In this review article, we briefly review the applications of SMLM in quantitative biology, and also the challenges involved and some of the solutions that have been proposed. Due to its advantages in labeling specificity and the relatively low overcounting caused by photoblinking when photo-activable fluorescent proteins (PA-FPs) are used as labels, we focus specifically on Photo-Activated Localization Microscopy (PALM), even though the ideas presented might be applicable to SMLM in general. Also, we focus on the following three quantitative measurements: single molecule counting, analysis of protein spatial distribution heterogeneity and co-localization analysis.

1. Introduction

With the invention of single molecule localization microscopy (SMLM) [1–3], it has become possible to extend the advantages of fluorescence microscopy beyond its diffraction limited spatial resolution of about 200 nm. This provides the possibility of resolving organelles or even single molecules with an order of magnitude better resolution, in multiple color channels and in 2D as well as 3D. Recent reviews on the updates on the technology and its uses can be found in [4,5]. SMLM can potentially be used for quantitative measurements [6,7], e.g., in counting the number of molecules of a protein specie [8] and stoichiometry estimation of protein complexes [9–11], characterizing the spatial distribution of a protein specie [12–15], estimating the co-localization or co-clustering between organelles and also single molecules (SM) [16–19], estimating the relative positions of various components in a protein complex with high precision [20,21], and estimating the diffusion coefficients by means of single particle tracking (SPT) in a dense sample [22,23]. Two basic variants of SMLM are Photo-Activated Localization Microscopy (PALM) and STochastic Optical Reconstruction Microscopy (STORM). The former uses fluorescent proteins for labeling (called photo-activable fluorescent proteins, PA-FPs), whereas the latter uses organic dyes. Since the usage of fusion proteins used in PALM provides comparatively high specificity labeling as against immunolabeling (the typical labeling technique used for STORM), and since the phenomenon of photoblinking for PA-FPs is minimal (as against the photo-switchable organic dyes used in STORM, which typically blink 10 times or more before irreversible photobleaching [24]), PALM appears to be better suited for quantitative studies, and for this reason forms the focus of this article even though many of the ideas presented are applicable to SMLM in general. Yet, quantitative analysis with PALM is plagued by several sources of errors [7,70], including that of a limited detection efficiency of label molecules in the range of 40–60% [16,25], a localization uncertainty in the order of 20–50 nm [26,27], overcounting in the range of 100% due to reappearance of label molecules due to photoblinking [15,28–30], errors in labeling, a sample drift in the order of 50–100 nm [1,31] and in the case of multi-color imaging, registration errors [16].

This review is divided into two parts. The application of SMLM has brought new discoveries in varied biological fields such as cell biology, neuroscience, microbiology and molecular genetics. First, we provide a bird's eye view of the applications of quantitative SMLM in these fields, focusing on the biological perspective. Then, with the help of cartoon figures, we explore in detail the challenges

* Corresponding author.
E-mail address: aleksandra.radenovic@epfl.ch (A. Radenovic).
[1] Current address: Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy.

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that are present in the use of SMLM, and specifically PALM, for quantitative experiments. We focus specifically on three specific quantitative applications of PALM: single molecule counting, analysis of protein spatial distribution heterogeneity and co-localization analysis. We also provide a brief summary of the methods that have been presented in the field to resolve the challenges presented.

2. Quantitative SMLM and biology

The possibility to quantify the number of proteins within biological assemblies and to characterize the protein spatial distribution has permitted to determine protein stoichiometry and distribution in signaling complexes. As a demonstration of this aspect, some groups, including ours, have analyzed the existence of protein aggregates such as oligomers and clusters for signaling receptors as G protein-coupled receptors (GPCRs), asialoglycoprotein receptors and RAS signaling molecules [9,10,32].

For the β2 adrenergic receptors, by means of quantitative cluster analysis, we found that the receptors are partially organized in mini-clusters only in the cardiomyocytes like-cells but not in other cell lines, and these oligomers are not lipid raft related but depend on actin cytoskeleton integrity (Fig. 1) [33]. Importantly, this conclusion was quite different from a similar report that was obtained using a different method named near-field scanning optical microscopy (NSOM) [71], as a demonstration of a better precision of PALM over other techniques. Receptor oligomers were not affected by the addition of different ligands, indicating that the receptor is already pre-associated before activation and is not related to receptor basal activity. In contrast, in a study by Renz et al. that made use of quantitative single molecule counting, it was shown that assembly of asialoglycoprotein receptors into homo- and hetero-oligomeric structures is dictated by exogenous ligands leading to the internalization of one receptor complex over another complex [9]. In this case, the authors used quantitative PALM together with ensemble Förster Resonance Energy Transfer (FRET) imaging. This experimental strategy has demonstrated the strong synergy that exists between these two different techniques combining the powerful sensitivity of FRET to detect receptor proximity with the capability to obtain direct visualization of receptor oligomers with PALM. A similar approach was also successfully applied to study another strategic protein in the RAS signaling, named RAF [10]. By means of cluster analysis, the authors showed how RAF exists between an inactive monomeric state in the cytosol and a multimeric condition at the cell membrane when activated. Together, these results confirmed the importance of dimers and oligomers formation in RAF signaling, even though the precise biological role of these different multimeric states is yet to be determined.

Another relevant consequence of the introduction of SMLM has been a better definition of biological structures in the nanometer range. This has been particularly true in the neuroscience field whereas the morphology of neurons composed by dendritic spines and synapses is not perfectly suitable for confocal microscopy. For example, imaging presynaptic and postsynaptic scaffolding proteins in glomeruli of the mouse olfactory bulb using STORM, Dani et al. showed distinct punctate patters that were not resolved by conventional fluorescence image [34]. They quantified various morphological parameters, and were able to distinguish the presynaptic Bassoon and postsynaptic Homer1 clusters. In this line of research, another group studied, by means of cluster analysis, the postsynaptic density (PSD) organization in live rat hippocampal neurons [35]. PALM was able to localize scaffolding nanodomains of PSD-95 enriched preferentially of AMPA receptors compared to NMDA receptors. This post-synaptic architecture could be relevant for the amplitude of postsynaptic currents, suggesting the mechanism of PSD in regulating the strength and plasticity of the glutamatergic transmission. For the optimization of cell morphology measurements in living cells using Single-Particle Tracking PALM, it has been shown using Monte-Carlo simulations how some technical parameters such as the length of the excitation pulse can influence the imaging of spine and spine neck morphology in living neurons, making them erroneously thinner when imaged using a longer excitation pulse [36].

SMLM was also applied to study exocytosis in different cell types, such as chromaffin cells. In PC12 cells, PALM was able to determine the size of clathrin coated pits during reuptake of vesicular acetylcholine transporters [37]. In contrast to what was found with confocal microscopy, Bar-On et al. used PALM to demonstrate that syntaxin1 and SNAP-25 clusters have a weak co-localization in PC12 cells [38]. Additionally, PALM helped to establish that clustered SNARE proteins are not involved in large dense core vesicles (LDCV) in the fusion process [39].

Another field in biology that has received attention for SMLM is microbiology, in particular for the study of bacteria and viruses. Because of the size of these microorganisms, the super resolution methods are suitable for revealing the details of their sub-cellular structures. For example, Ptacin et al., studying the partitioning (Par) apparatus that guides centromere segregation, were able to

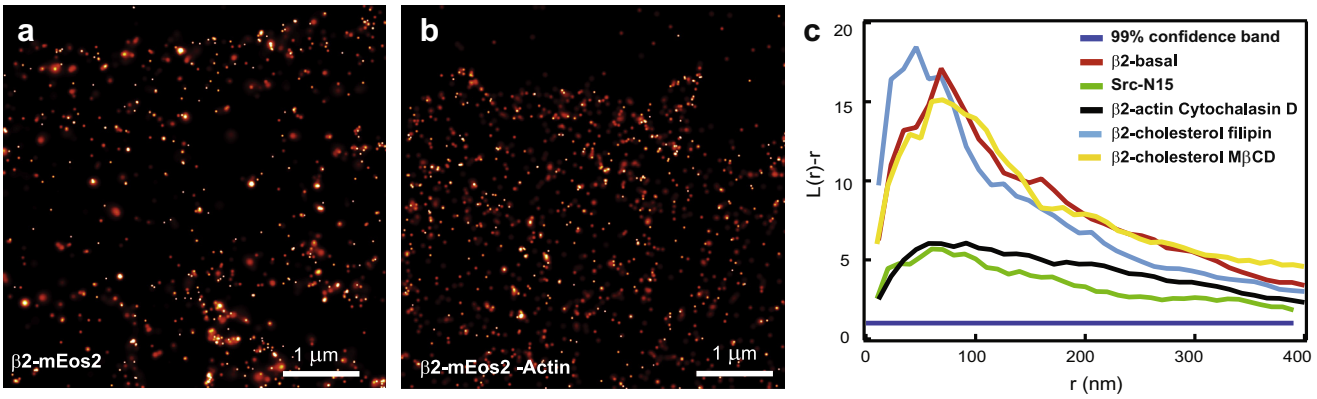

Fig. 1. PALM images and cluster analysis of β2-mEos2 on the plasma membrane of H9C2 cells after cholesterol sequestration or actin microfilament disruption. (a–b) PALM images in total internal reflection fluorescence geometry of β2-mEos2 on the plasma membrane of fixed H9C2 cells in basal condition (a), and after actin cytoskeleton disruption (b). (c) The degree of clustering for the experiments shown was determined by Ripley's K function analysis and $L(r) - r$ parameter that displays the magnitude of deviations from a random distribution as positive y values (normalized to 99% confidence interval). Data are representative of experiments that were repeated at least three times. Cholesterol inactivation was obtained by preincubating cells with filipin for 30 min at the concentration of 12 μg/ml or MβDC with a preincubation of 30 min at the concentration of 5 mM, while actin microfilaments disruption was performed by preincubating for 30 min with cytochalasin D at the concentration of 2 μM.