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Single Molecule Localization Microscopy for Studying Small Extracellular Vesicles

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Small extracellular vesicles (sEVs) are 30–200 nm nanovesicles enriched with unique cargoes of nucleic acids, lipids, and proteins. sEVs are released by all cell types and have emerged as a critical mediator of cell-to-cell communication. Although many studies have dealt with the role of sEVs in health and disease, the exact mechanism of sEVs biogenesis and uptake remain unexplored due to the lack of suitable imaging technologies. For sEVs functional studies, imaging has long relied on conventional fluorescence microscopy that has only 200–300 nm resolution, thereby generating blurred images. To break this resolution limit, recent developments in super-resolution microscopy techniques, specifically single-molecule localization microscopy (SMLM), expanded the understanding of subcellular details at the few nanometer level. SMLM success relies on the use of appropriate fluorophores with excellent blinking properties. In this review, the basic principle of SMLM is highlighted and the state of the art of SMLM use in sEV biology is summarized. Next, how SMLM techniques implemented for cell imaging can be translated to sEV imaging is discussed by applying different labeling strategies to study sEV biogenesis and their biomolecular interaction with the distant recipient cells.

1. Introduction

Extracellular vesicles (EVs) are lipid bilayer-bound vesicles secreted by all cell types into the extracellular space. EVs are broadly classified into three main types, viz., exosomes (30–150 nm), microvesicles (150–1000 nm), and apoptotic bodies (1–5 µm).[1–3] EVs are very heterogeneous, and the Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018) guidelines suggests that EV researchers use the term small EVs (sEVs) for vesicles that are less than 200 nm in diameter.[4] sEVs contain many essential cargo biomolecules such as nucleic acids (DNA, mRNA, microRNA), proteins, and lipids.[1,3,5] sEVs have emerged as a functional mediator for communications between cells in health and disease.[1,6] sEVs derived from tumor cells contain disease-specific proteins, RNA, and double-stranded DNA (dsDNA), thereby representing the disease state and progression.[1,2,7–9] Especially in tumor microenvironment, sEVs transfer their cargo from the tumor to stromal cells.[10,11] sEVs play pathophysiological roles in many other diseases, including neurodegenerative diseases, and various infections. They transmit important biomolecules that regulate many biological processes and influence the immune system.[1,12–15]

In the last decade, studies dealing with sEV biogenesis pathways and the role of sEVs in health and disease have grown exponentially. However, exploring the released sEVs and interaction of their cargo with cellular biomolecules in the distant recipient cells is significantly hampered by the lack of microscopy studies using improved imaging technology. Most of the current microscopy studies involving sEVs use conventional confocal microscopy to generate either 2D or 3D reconstruction images using Imaris.[11,16–22] Although these images provide the information

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related to sEV communication, they fail to resolve the precise localization and interaction of sEV-associated cargo with cellular biomolecules below the range of ≈200–300 nm. Using confocal imaging, we have recently shown that EV-DNA derived from acute myeloid leukemia (AML) interacts with bone marrow derived mesenchymal stromal cells (BM-MSCs). However, using 2D confocal imaging, we could not discern precisely how AML EV-DNA communicated with which cellular biomolecules in BM-MSCs.[11] Therefore, it is necessary to apply super-resolution microscopy (SRM) with an innovative labeling strategy, which enables an optical resolution of nanometer range to study the biomolecular interaction of sEVs with various components in the recipient cells in real time.

Among various SRM techniques (**Figure 1**), single molecule localization microscopy (SMLM) has been mostly used in EV research, since SMLM provides excellent resolution and higher signal-noise ratio, which helps to spatially quantify the arrangement of single molecules.[23,24] Nevertheless, EV researchers mostly implemented SMLM only for sEV characterization by labeling the common sEV markers using antibodies linked with different fluorophores. In general, studies that investigated the functional role of sEVs using SRM in the distant recipient cells are very limited. In this regard, we attempted to study the interaction of sEV-associated DNA cargo with cellular components in the distant recipient cells, using SMLM. However, we faced several challenges due to the commonly used fluorophores. In this review, based on our experience, we discuss the pitfalls and limitations of existing labeling fluorophores and the necessity to apply alternative fluorophores with exceptional blinking properties in SMLM sEV imaging. Further, we summarize how future advances in live- and fixed- cell imaging of sEVs at nanoscale resolution can address critical questions in sEV biology, such as the packaging of different biomolecular cargo into sEVs, uptake of individual sEVs, and the molecular interaction of sEVs with specific cellular compartments.

2. Single Molecule Localization Microscopy (SMLM)—Current State of the Art and Limitations

2.1. Principle of SMLM

Images acquired through a conventional fluorescence microscopy are a blurred presentation of the object under microscope. The image of a single molecule appears as a central peak that spans around 200–300 nm in width, usually defined

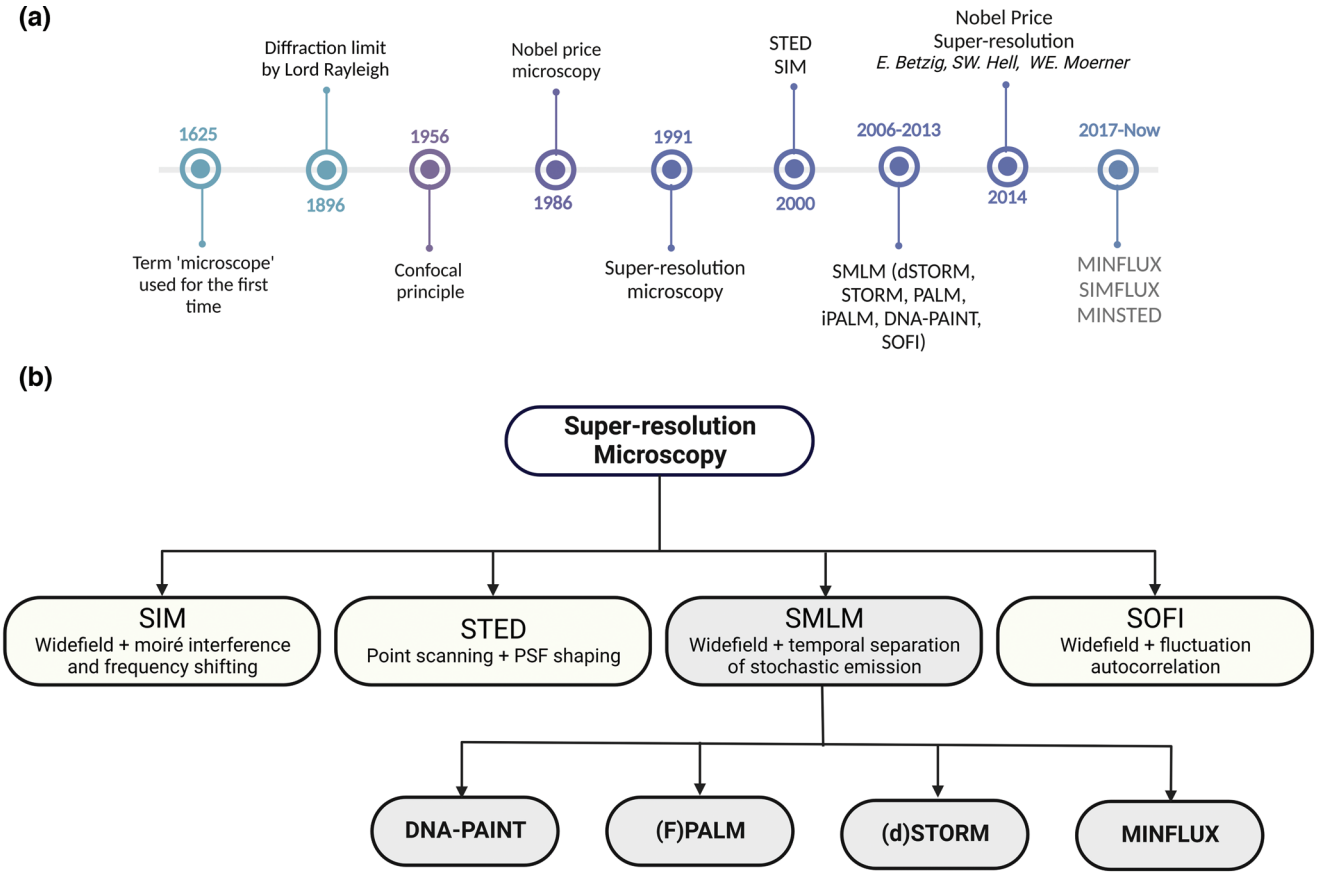

Figure 1. Methods of super-resolution microscopy. a) Major breakthrough in the light microscopy field. b) The SMLM methods are based on sequentially imaging a sparse subset of fluorophores and computing their positions from the diffraction patterns obtained. SMLM: single-molecule localization microscopy; STED: stimulated emission depletion microscopy; SIM: structured illumination microscopy. (F)PALM: (fluorescent) photoactivated localization microscopy (PALM); (d)STORM (direct) stochastic optical reconstruction microscopy; DNA-PAINT: DNA-based point accumulation for imaging in nanoscale topography; smFRET: single-molecule Förster resonance energy transfer; MINFLUX: MINimal photon FLUXes.

as point spread function (PSF) (**Figure 2**b). This limited spatial resolution is caused by the diffraction of light into the far field known as the diffraction limit of light microscopy. In other words, objects closer than 200–300 nm, for example sEVs proteins, could not be resolved by light microscopy approaches. The development of super-resolution microscopy (SRM) techniques, specifically single-molecule localization microscopy (SMLM) and stimulated emission depletion (STED) microscopy, has widely overcome the diffraction limit.[25,26] The importance of this technology can be gauged by the Nobel prize in Chemistry (2014) awarded to Eric Betzig, Stefan W. Hell, and William E. Moerner for developing this technology (Figure 1a). Most of these SRM approaches are based on the time domain (reconstruction from a series of conventional resolution images registered at different time frames). All these developed SRM techniques share the same basic principle: the adjacent fluorophores in the diffraction limit area are discernable during registration and their coordinates are identified with high precision.[27] While super-resolved structured illumination microscopy (SR-SIM)[28] and STED[29] benefit from the approaches of periodic illumination patterns such as excitation beams and reversible saturable optical fluorescence transitions (RESOLFT) of fluorophores, SMLM and recently developed second-generation optical super-resolution imaging techniques, including MINFLUX,[30] SIMFLUX,[31] and MINSTED,[27] are based on the adoption of photo-switchable/blinking fluorophores (Figure 2c). SMLM methods, including photoactivated localization microscopy (PALM),[32] stochastic optical reconstruction microscopy (STORM),[33] ground-state depletion microscopy (GSDIM),[34] and direct STORM (dSTORM),[35] are based on the time domain and allow an optical resolution down to the range of a few nanometers (Figure 1b).

In SMLM, a sequence of optically isolated fluorophores is recorded and therefore requires multiple image frames (Figure 2). In each frame, the fluorophore positions are determined to reconstruct a super-resolution image by creating a joint localization map (Figure 2d). The required number of image frames depends on the structures of interest, the labeling density and the switching behavior of the fluorophore. To faithfully reconstruct the structure of a certain size

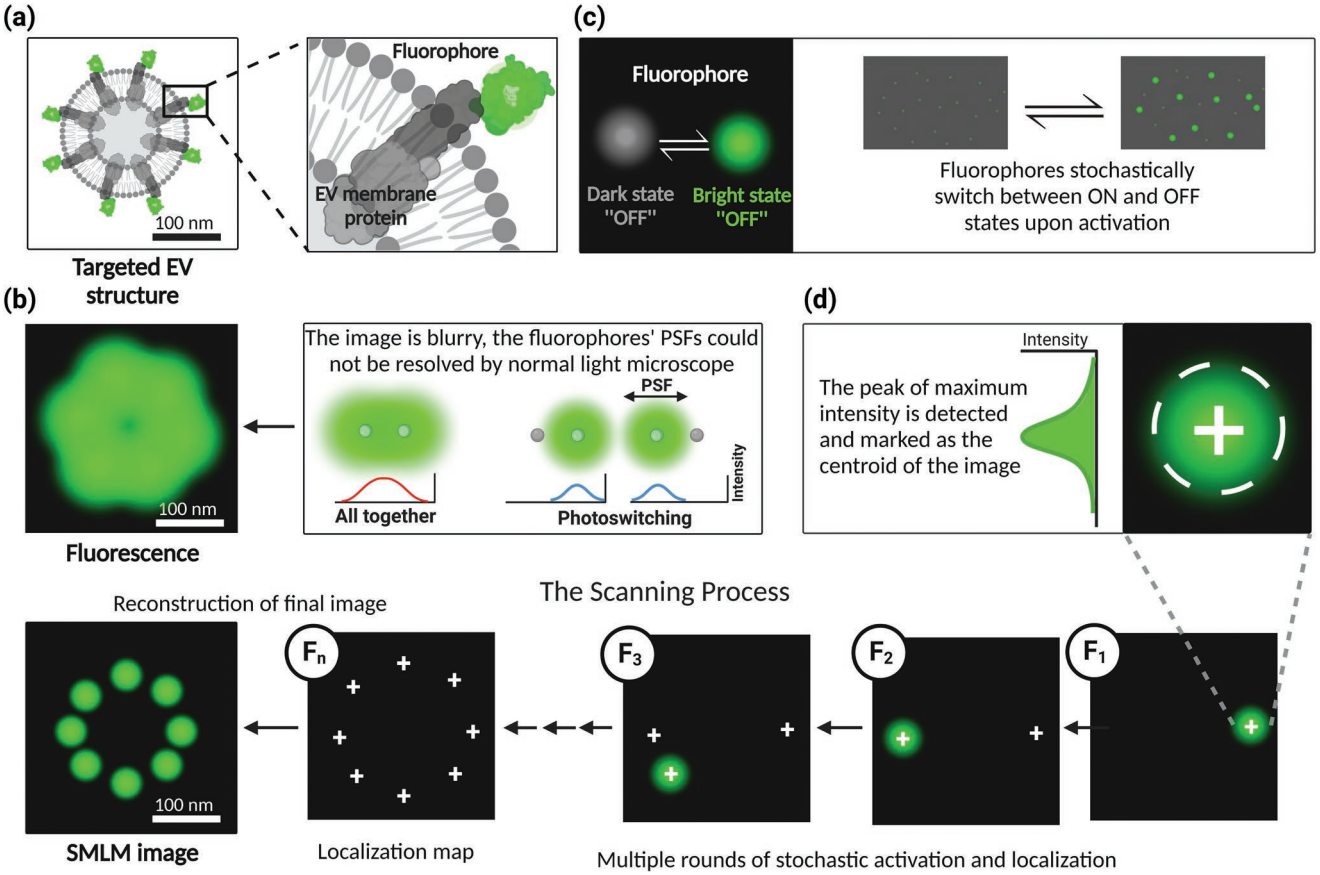

Figure 2. Principle of single-molecule localization microscopy (SMLM). a) Structure of sEVs labeled with a nanobody conjugated to a photoswitchable fluorophore. b) A fluorescent molecule (green dot) imaged with a light microscope appears as a fuzzy spot conventionally known as the point spread function (PSF) that extends over multiple pixels in the acquired image. PSFs from simultaneously emitting fluorophores overlap when the distance that separates them is smaller than the PSF, blurring the structure. c) SMLM usually exploits the fact that fluorophores stochastically switch between an active ("ON") state and one or more inactive ("OFF") states. d) In SMLM, the peak of maximum intensity is detected and marked as the image's centroid to determine the exact position of the emitting fluorophore. In each image frame, only one molecule emitting fluorescence is detected within an object region corresponding to the conventional resolution limit of the microscope system used. Assignment of all the individual molecule positions to one joint localization map provides the spatial distribution of sEVs with enhanced optical resolution. Localization map used to reconstruct nanostructural information of the individual sEV studied in its cellular context.

according to the Nyquist–Shannon sampling theorem, one needs to sample the structure of interest with a frequency that is at least 2 times higher than its spatial frequency.[36] Stochastic fluorescent molecules allow an exact spatial and qualitative assignment due to the temporally different fluorescence activation. The activation of individual fluorescence at different time points (bleaching/blinking) enhances the quality of resolution. As shown in Figure 2d, the packaging of different biomolecular cargos into sEVs and the molecular cargo interaction of sEVs in the distant recipient cells can be analyzed by implementing SMLM (Figure 2a,d).

For the study of biomolecular interactions at an optical resolution below ≈10 nm, SMLM is now available in a variety of commercial and custom-made variants, depending on different fluorophores and ways to switch between ON and OFF states.[37,38] For example, to study three biomolecules tagged with different fluorescent proteins and interacting at the distance range of 50 nm, the biomolecules may be fluorescence-labeled with three "spectral signatures," which can be localized independently from each other, due to their differences in the absorption/emission spectrum.[39,40] On the other hand, the optical isolation of single molecules in the time domain using wide-field fluorescence microscopy may be achieved either by using different fluorescence lifetimes,[41,42] or by switching the molecular fluorescence between different emission modes ("blinking"). This latter method was first proposed by the group of Rainer Heintzmann using quantum dots in 2005;[43] shortly thereafter, Betzig et al., 2006 applied this concept experimentally to successfully image GFP in SMLM with an optical resolution ranging a few nanometers.[32] In general, SMLM substantially reduces the resolution problem of simultaneous signal acquisition and characterizes the spatial arrangement of single molecules.

2.2. SMLM for sEV Imaging

The role of sEVs in intercellular communication in various disease settings, especially cancer, and their application for therapeutic development have been actively investigated. In order to understand the role of sEVs in various diseases, it is vital to label and image sEVs right from their release from the parental cells until their spatial distribution and bio-molecular interaction in the distant recipient cells. Both in vitro and in vivo imaging of sEVs play a significant role in molecular medicine by helping the EV researchers to understand the biogenesis of sEVs and their molecular function in various disease contexts.[44,45] Bioluminescence- and fluorescence-based sEV labeling has been used mainly to detect the sEVs through the conventional fluorescence microscopy. Due to their relatively small size, ranging from 30–200 nm, it is challenging to image sEVs at the single-vesicle level and to directly track the process of sEV communication within the recipient cells. Recently, Verweij et al., 2021 discussed the advancements in microscopy techniques combined with novel labeling strategies to study sEVs in vivo in their physiological environment and at the single-vesicle level.[46]

Until now, most of the EV researchers used SMLM technique only for EV characterization, but not for functional studies. Because the current EV characterization techniques have poor sensitivity to detect sEVs below 50 nm. In SMLM, they mostly used dSTORM (Direct Stochastic Optical Reconstruction Microscopy) based imaging since dSTORM exhibits excellent photoswitching and minimal photobleaching.[39,40] Chen et al., 2016 were the first one to perform intracellular tracking of cancer-derived exosomes using STORM/PALM imaging.[47] In their study, antibodies conjugated with different fluorophores were used to label exosome markers, and a fluorescent dye, CM-Dil was used to label the exosome membrane. Following this, Nizamudden et al., 2018 performed dSTORM-based imaging of DiD-labeled mesenchymal stromal cells (MSC)-derived sEVs.[48] However, DiD is a lipophilic dye similar to other commonly used EV labeling dyes such as PKH and CFSE, which have a strong tendency to form aggregates, and it also labels non-EV contaminants such as lipoproteins.[49] Following this, many EV researchers performed dSTORM-based imaging of sEVs by labeling the EV canonical markers such as CD9, CD63, CD81, and TSG101 using the corresponding antibodies conjugated with different fluorophores.[50–55] Recently, STORM-based imaging was performed to reveal the heterogeneity in EV secretion by human macrophages.[56] The details of all the published studies based on SMLM for EV and their cargo characterization are furnished in **Table 1**.

Table listing the information such as source of sEVs, method of sEV isolation, details of sEV/sEV cargo imaged, and SMLM technology involved in SMLM sEV studies. Abbreviations: PEG- Polyethylene Glycol, UF- Ultrafiltration, SEC- Size Exclusion Chromatography, TFF- Tangential Flow Filtration.

2.3. Other SRM Methods for sEV Imaging

In addition to SMLM, many other SRM techniques have been employed to image sEVs. Dabrowska et al. 2018 showed the uptake of PKH26^{+} sEVs by human bone marrow mesenchymal stromal cells using super-resolution structured illumination microscopy (SR-SIM).[57] SR-SIM uses interference-generated light patterns, which enables this technique to extract information with higher resolution.[58] On the other hand, Mighty et al., 2020 recently deciphered the function of EV-RNA in the recipient cells using SR-SIM. However, they labeled EV-RNA externally, using SytoRNA Select (Invitrogen), which labels RNA derived from both metabolically and nonmetabolically active cells.[59] Conversely, our group used EdU (5-ethynyl-2'-deoxyuridine) to label the DNA derived only from metabolically active cells, an approach that could be utilized to ascertain the biological role of EV-DNA in various disease models. Then, we employed SMLM to reveal the precise localization of EV-DNA along with various cellular proteins at the nanoscale level with enhanced resolution and high accuracy. However, as previously mentioned, we could not measure the level of interaction of EV-DNA with cellular proteins due to the bleaching of the GFP signal caused by copper ions present in the Click-iT EdU staining kit.[11]

Single-molecule fluorescence microscopy, using Förster resonance energy transfer (FRET) as a structure-sensitive probe, is an effective technique to detect sEVs at single-vesicle level and to study the interaction of sEVs with other cellular

Table 1. Super-resolution microscopy (SRM) for imaging small extracellular vesicles (sEVs).

sEVs source	sEVs isolation	sEVs/sEV cargo	SMLM technique	Refs.
Breast cancer cell line (SKBR3) and cervical cell line (HeLa)	Exosome collection kit (System Biosciences)	$CD63^+$sEVs and CM-Dil stained sEVs	STORM/PALM	[47]
Mouse mesenchymal stem cells	exoEasy Maxi Kit	DiD-labeled sEVs	dSTORM	[48]
KW10 glioblastoma cell line	PEG precipitation	$CD63^+$/$TSG101^+$ sEVs	dSTORM	[50]
Human osteosarcoma cells (U2OS)	TFF + PEG precipitation	$CD9^+$/$CD63^+$/$CD81^+$ sEVs	dSTORM	[51,53]
Bone marrow, adipose tissue, and umbilical cord MSC-EVs	Sequential ultracentrifugation	$CD9^+$/$CD63^+$/$CD81^+$ sEVs	dSTORM	[52]
SARS-CoV-2 spike-transfected and nontransfected HEK293T cells	Sequential ultracentrifugation	$CD9^+$/$CD63^+$/$CD81^+$ sEVs	dSTORM	[54]
Human glioblastoma cells	Total Exosome Isolation Kit (Thermo fisher Scientific)	$CD63^+$/$TSG101^+$ sEVs	dSTORM	[55]
Human macrophages	Sequential ultracentrifugation	$CD9^+$/$CD63^+$/$CD81^+$ sEVs	STORM	[56]
HEK293T-CD63-GFP cells	TFF+SEC+UF	$CD63^+$ sEVs and EV-DNA	dSTORM	[11]

components that are within nanometer range. Zhang et al.,[60] and Thorsteinsson et al.,[61] developed an assay using FRET fluorophores to quantify the extracellular vesicles and other vesicles of complex composition. In these studies, they demonstrated that FRET assay is advantageous over the commonly used EV characterization technique such as Nanoparticle Tracking Analysis (NTA), because NTA has the limit of detection only 50–70 nm. Combining FRET fluorophores used in this study with single-molecule fluorescence microscopy (smFRET) will lead to a powerful biophysical technique in future for the study of EV structural dynamics, for instance, measuring the distance between two EV cargo biomolecules that are within 1–10 nm.[62,63]

2.4. Advantages and Disadvantages of SMLM over Other SRM Methods

Although SIM technique has simple sample preparation steps and ease of operation and causes less photodamage, it provides only ≈100 nm resolution compared to SMLM, which has a resolution of about ≈20–30 nm that is suitable for imaging sEVs.[64] In contrast, STED provides better resolution (≈50 nm) than SIM; however, it causes high photo damage ultimately affecting the super-resolved image.[65] Further, SMLM and STED require high laser power to image live cells for fast turnover and rapid imaging, leading to photobleaching and phototoxicity.[66] On the other hand, SIM requires only minimal laser power to detect fluorescent photons with high efficiency.[28] Despite STORM/dSTORM SMLM techniques are commonly used for EV imaging, one of the other SMLM techniques, "MINFLUX," emerged as an efficient one since it provides resolutions of 1–3 nm both in lateral and axial planes. Not only that, MINFLUX requires less laser intensity for the fluorophore excitation since in MINFLUX fluorophores switch between on/off states as in PALM/STORM as well as simultaneous illumination with a doughnut-shaped excitation (rather than depletion) beam.[67] Considering these improvements, MINFLUX could serve as an efficient SMLM technique to image sEVs. In addition, recently developed technique such as reversible saturable/switchable optically linear (fluorescence) transitions (RESOLFT) obtained better resolutions with minimal excitation power, which makes it suitable for live-cell imaging.[68,69] ven though SMLM has some disadvantages compared to other SRM methods, they do have advantages that can be really useful for sEV imaging like easy implementation, ease of operation, and, importantly, optimized blinking buffers and fluorophores for EV quantification.

3. Perspectives of SMLM for sEV Imaging

3.1. SMLM Fluorophores for sEV Imaging and Their Limitations

SMLM fluorophores are usually classified according to the switching mode between ON and OFF states, as summarized in **Figure 3**a and thoroughly discussed by Lelek et al.[66] Photoswitchable fluorophores switch reversibly (multiple blinking for a single fluorophore) between ON and OFF states, whereas photoactivatable fluorophores irreversibly shift from to ON state either spontaneously or upon activation by UV light. Photoconvertible fluorescent proteins switch also irreversibly from one spectral state (color) to another upon irradiation. Spontaneously blinking dyes exploit a reversible, pH-dependent chemical reaction to enable SMLM at a defined pH in an aqueous solution without requiring a photoswitching buffer. Synthetic dyes and fluorescent proteins are both utilized for SMLM imaging. However, presently organic fluorophores are most utilized compared with fluorescent proteins, inorganic quantum dots, and carbon dots, due to the small size, excellent blinking properties, low toxicity, and biocompatibility.[70] Photoactivatable/-switchable fluorescent proteins are mostly suitable for live-cell applications, despite their premature bleaching or incomplete labeling that can hamper the structural resolution.[70] For live-cell imaging, fluorophores should have the following properties: i) good cell-membrane permeability and solubility to improve the labeling efficiency and reduce the background signal, ii) biocompatibility and low toxicity, iii) long absorption and emission wavelengths and low laser density to reduce the laser effect on cell physiology, and iv) specific targeting ability toward the molecules of interest.[70] In addition to these properties, it is important that the fluorophores should exhibit excellent switching properties (high photon numbers per switching events) and low duty cycles to obtain high imaging resolution. However, in order to exhibit excellent blinking properties,

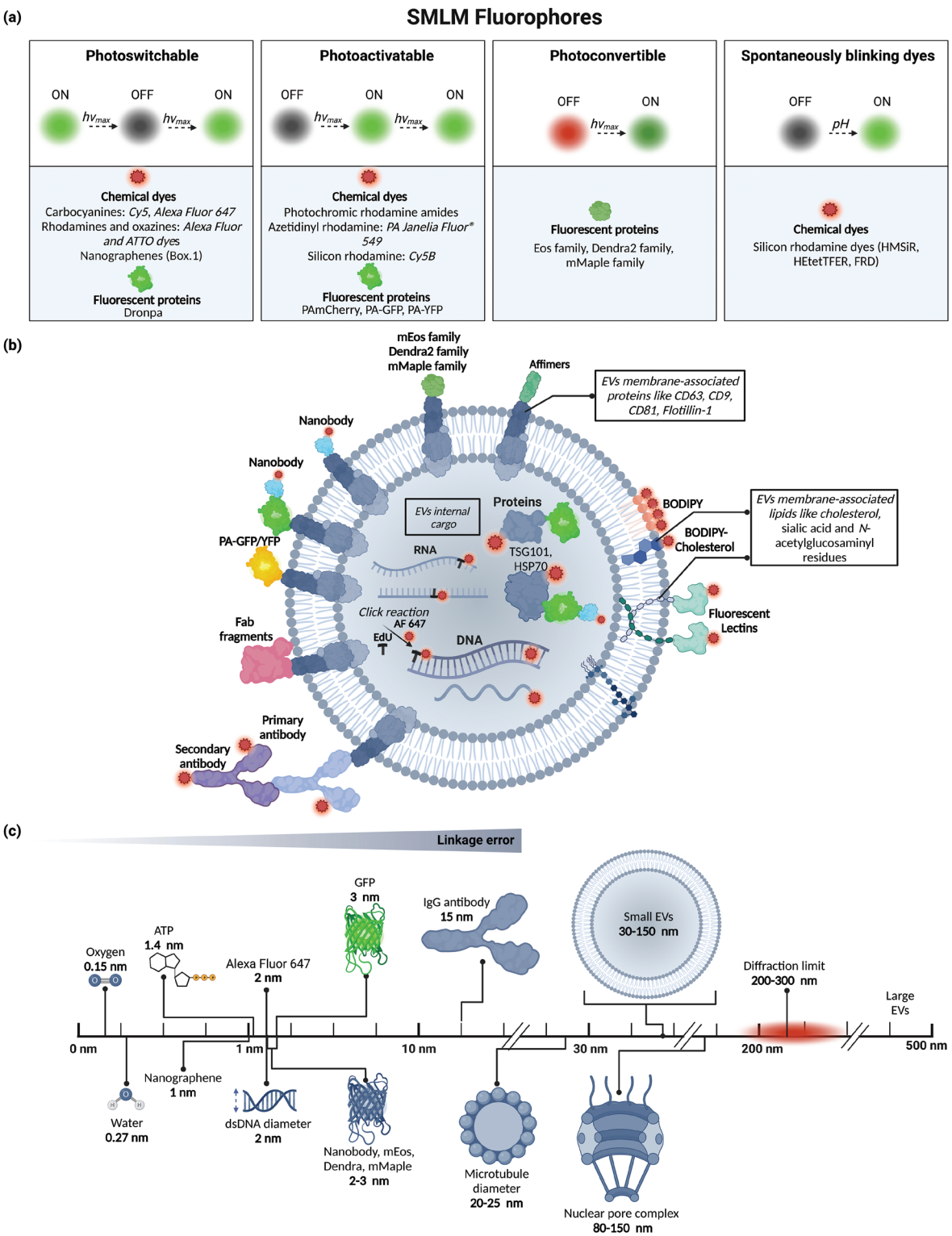

Figure 3. Classification of SMLM fluorophores and labeling strategies for sEVs. a) Single-molecule localization microscopy (SMLM) fluorophores fall into four classes (plus the temporarily binding dyes used in the techniques of DNA point accumulation in nanoscale topography, such as DNA-PAINT). An example of a fluorophore is included in each class. "ON" fluorophores are indicated in green. b) Different labeling approaches for imaging sEVs with SMLM. sEVs are highly heterogeneous in terms of size. To reduce the linkage error, labeling can be done with: camelid nanobody combined with or without GFP; fragment antigen-binding (Fab); protein tag or genetically encoded protein, such as Eos family; fatty acid analogs conjugated to photoswitchable fluorophores like BODIPY-C12; fluorescent lectins that target sEVs membrane-associated sialic acid and *N*-acetylglucosaminyl residues; nucleotide analogs like EdU, which the click reaction can detect. c) Spatial scales of chemical fluorophores and fluorescent proteins that can be utilized in SMLM. h*ν*, irradiation at the absorption maximum.

most of the organic fluorophores need some additives for the imaging buffer that arecompatible with live cells. The lack of availability of such additives greatly influence the applicability of live-cell SMLM.

An increase in temporal resolution reduces the structural resolution and vice versa. Additionally, SMLM uses relatively high excitation intensities over an extended period of time and thereby causes phototoxicity eventually leading to cell death. Organic dyes are limited in cell permeability and require special imaging buffers that enable molecular blinking. These buffers might not be compatible with live cell microscopy. To overcome these limitations and to increase imaging speeds, as well as to reduce phototoxicity, several approaches are adopted. As the imaging speed of SMLM strongly depends on the blinking properties of the fluorophores, it is aimed to optimize the photon statistics of molecular blinking toward fast and bright signals at low excitation powers.[71] In this context, alternative fluorophores such as nanographenes might be helpful.[72] An additional option to reduce the phototoxicity would be to enhance the localization precision obtainable at a given photon yield by structured illumination patterns, such as STED-like "Donuts" in MINFLUX-based SMLM,[30] or harmonic illumination patterns in wide-field SMLM.[31,73,74]

Other approaches use artificial intelligence to improve imaging speeds. This is done either by improving the localization of individual molecules in high-density images[75–77] and thereby reducing the overall number of image frames but keeping the total number of localized molecules in an SMLM image the same, or by reconstructing super-resolution images from sparse localization data.[78] Both the methods rely on generating a priori knowledge, stored in a pertained neuronal network, to reduce the number of image frames needed for generation of super-resolution images with high fidelity.

Entirely different approaches can be tried to slow down the observed processes rather than speed up the imaging process. These methods freeze the cells on the microscope stage and arrest them in their current state. Extended imaging is then performed on the frozen cells. After imaging, cells are thawed and "brought back to life."[79]

3.1.1. Next-Generation SMLM Fluorophores

For SMLM, quantum dots (QDs) exhibit more photostability and brightness than organic dyes, making them promising alternatives for biological imaging.[80,81] Quantum dots such as ZnSe and Mn-doped ZnSe nanocrystals,[82] CdSe [83,84] have been successfully utilized in different super-resolution techniques. The inorganic perovskite nanocrystals (CsPbX3 NCs, X = Cl, Br, and I) have recently emerged as an intriguing surrogate to the conventional light emitters due to the low-temperature solution processability, high photoluminescence quantum yield, narrow full width at half-maximum, and vast region tunable emission spectra.[85–87] Especially, Yang et al. 2022 were able to distinguish two closely spaced exosomes (with a gap of 54 nm) stained with CsPb(Cl(1–*x*)/Brx)3@PEG by SMLM.[88] However, large size (≈10 nm) and high on-off duty cycle,[89] the toxicity of QDs containing heavy metal atoms,[90] and rigorous synthesis conditions[91,92] limit their use as reliable less invasive probes for biological imaging. Therefore, upgraded versions of QD probes with the same outstanding photophysical properties but with easy synthesis process and less toxicity are needed for next-generation SMLM bioimaging.

In this regard, nanoscale graphene quantum dots (also called nanographenes) with well-defined quantized energy levels have recently been proposed as an environmentally friendly alternative to QDs and carbon dots.[93] Nanographenes exhibit outstanding photophysical properties, such as intrinsic blinking even in the air, excellent fluorescence recovery, and stability over several months for SMLM imaging.[72] Nanographenes are emerging as another class of superior photoswitchable fluorophores due to their small size (≈1 nm) and low toxicity.[72] Nanographenes correspond to nanoscale fragment structures of graphene, consisting of multiple fused benzene rings.[94] Atomically precise, molecular nanographenes can be synthesized through the methods of synthetic organic chemistry and obtained as a single product that can be characterized by NMR, mass spectrometry, and other conventional methods for organic compounds.[95,96] Molecular nanographenes with a variety of structures have been reported, demonstrating structure-dependent optical properties with well-resolved absorption and emission bands like those of organic dyes.[97] Similar to organic dyes, nanographenes can be covalently functionalized to prepare various derivatives, and the synthesis of water-soluble nanographenes has already been reported, which have been used in formation of self-assembled nanoarchitectures, bioimaging, and cancer sono-dynamic therapy applications.[98,99] The environment-independent blinking properties and chemical modification of nanographenes make them capable of labeling and imaging sEVs.[72,95]

3.2. SMLM Live-Cell sEV Imaging to Reveal sEV Cargo Loading Mechanism and Functional Role in the Recipient Cells

Resolving a structure of interest requires sampling of this structure with a frequency that is at least twice as high as its spatial frequency.[36] In simple terms, this means if one aims to image and resolve an individual sEVs of 30 nm in diameter, one would have to measure and precisely localize a molecule at least every 15 nm along one dimension, which corresponds to 66 molecules μm^{-1}, or in three dimensions to 290 000 molecules μm^{-3}. This simple assumption neglects any sources of noise and therefore only provides a lower bound for the minimal number of localizations.[100] A priori knowledge about the structure of interest can be used to substantially reduce the number of required localizations. It might not be necessary to achieve such high labeling densities and high number of localizations if it is known that sEVs are spherical in shape. Nevertheless, multiple localizations and hence multiple images will be required to reconstruct a super-resolved image with a high structural resolution. Therefore, live-cell SMLM can only be performed for structures that change slowly with respect to the time needed for image acquisition. For such structures, it is possible to image a sufficient number of fluorophores to have a faithful reconstruction of the structure before it changes over time.[101–104]

Despite the wide variety of fluorophores that exist in the market, choosing the suitable labeling strategy for SMLM live-cell sEV imaging is always challenging due to the small size of sEVs. sEVs are nanovesicles (average size below the diffraction limit) that are released by cells via several mechanisms starting from the inward budding of the multivesicular body membrane to form tetraspanins (CD9, CD63, CD81) or ceramide rich regions.[3] During their journey of biogenesis, proteins, nucleic acids, and other cytosolic components are engulfed and enclosed within sEVs. Hence, structural characteristics and molecular content of sEVs allow us to combine different labeling strategies for SMLM imaging to study EV biogenesis and uptake dynamics. For example, sEVs can be labeled using a lipid dye or an epitope for EV surface proteins along with a dye targeting the intraluminal component of interest (Figure 3b).

Until now, only Chen et al. 2018 reported the imaging and tracking of exosomes and exosomal miRNAs simultaneously employing dual color SMLM in living cells, since sEV-miRNAs derived from stromal cells in the tumor niche influence tumorigenesis and metastasis.[105] They utilized molecular beacons to label HeLa-derived exosomes (isolated using ExoQuick-TC Precipitation) and two kinds of exosomal miRNAs (mir-21 and mir-31). Molecular beacons (MBs) are hairpin-shaped hybridization probes containing specific oligonucleotide sequence (target) with an internally quenched fluorophore whose fluorescence would be revived when it binds to a target oligonucleotide sequence. In general, MBs could be applicable for any target oligonucleotide sequence and therefore, MBs with specific EV-DNA or EV-RNA sequences would enable EV researchers to study the interaction of EV-DNA/EV-RNA in the recipient cells in real time. Although MBs provide greater single-molecule sensitivity and high signal to noise ratio, they often compete with endogenous DNA- or RNA-binding proteins for target binding and also require toxic intracellular delivery agents.[106] Therefore, the development of suitable fluorophores that can be applied in SMLM live-cell sEV imaging remains crucial. Nevertheless, scientists have successfully used some organic fluorophores in live-cell SMLM, which also can be potentially applied in SMLM live-cell sEV imaging.

3.2.1. sEV Membrane and Surface Protein Labeling

Shim et al. 2012 demonstrated photoswitchable capabilities of commonly used membrane fluorophores for plasma membrane (DiI, DiD, DiR), mitochondria (MitoTracker Orange/Red, Deep Red), endoplasmic reticulum (ER-Tracker Red), and lysosomes (LysoTracker Red).[107] They obtained super-resolution images of membrane structures in living cells labeled with spectrally distinguishable probes using imaging buffer with a spatial resolution of 30–60 nm and temporal resolution of 1–2 s. Photoswitchable BODIPY-based fluorophores have recently shown high efficiency in tracking lysosomes in SMLM live-cell imaging, which enables a versatile live-cell imaging of other subcellular structures, such as sEVs at the nanoscale level.[108,109] Despite the fact that many of the above-mentioned membrane dyes could be applied in live-cell sEV imaging by SMLM, the imaging was performed with the help of buffers containing oxygen scavenging systems additives, which are incompatible with live-cell study. Therefore, we need a special dye for live-cell imaging which has excellent blinking properties and the imaging can be performed with the buffer without any additive. For this, high-density environment-sensitive membrane dye (HIDE), based on spontaneously blinking dye silicon-rhodamine (HMSiR) was developed. This dye has the potential to label mitochondria, plasma membrane, filopodia, and endoplasmic reticulum to study their 2D dynamics without the need of additives and irradiation with UV light.[71,110] With these advantages, HIDE serves as a better choice for SMLM live-cell sEV imaging to study the interaction of sEVs with plasma membrane, mitochondria, endoplasmic reticulum, and lysosomes over different time frames.

Recently, Qiao et al. reported an acid-regulated self-blinking probe, LysoSR-549, which can achieve the switching of fluorescent and nonfluorescent dyes in acidic environment.[111] In this study, they utilized LysoSR-549 for long-term super-resolution imaging of lysosomes in live cells and illustrated different lysosome movement routes and interaction modes between lysosomes. Since LysoSR-549 is compatible with both fluorescent and nonfluorescent probes, any lysosomal EV marker (e.g., LAMP1) would be applied along with LysoSR-549 to demonstrate the interaction between lysosomes on sEVs content in recipient cells.

Indeed, membrane dyes can be added directly to the culture media (e.g., HMSiR) when cultivating cells for sEV isolation.[112] However, less is known about how cell labeling affects sEV release and function. On the other hand, membrane dyes have a long half-life compared to sEVs after cellular uptake, which can lead to misleading conclusions regarding sEV dynamics in the recipient cells.[113–115] Therefore, membrane dyes seem to be more suitable to label sEVs for short-term studies.

sEVs can also be tagged using epitopes that target sEV surface proteins such as tetraspanins and flotillin-1 (Figure 3b). However, use of the classical immunolabeling with primary and secondary antibodies introduces a large linkage error (Figure 3c), due to the large size of immunoglobulins.[116] For this reason, small proteins like GFP/YFP, nanobodies, and fragment antigen-binding (Fab) can be good surrogates. Another alternative is the use of photoswitchable fluorescent proteins such as the members of mEos family, Dendra2 family, and mMaple family, because in general fluorescence proteins are more appropriate for performing SMLM live-cell imaging.[117]

3.2.2. sEVs Intraluminal Cargo Labeling

In 2010, Wombacher et al. applied the photoswitchable organic fluorophore ATTO655 conjugated with trimethoprim (TMP) to label the histone H2B tagged with dihydrofolate reductase (eDHFR) and performed live cell SMLM under physiological conditions.[103] ATTO655 can achieve the conversion of fluorescent state "on" and nonfluorescent state "off" in the presence of oxygen and glutathione.

Altogether, the studies mentioned in this section demonstrated the power of SMLM to study how particular biomolecular cargo is loaded into sEVs and, importantly, the interaction of sEVs in live recipient cells under physiological conditions.

3.3. SMLM to Study the Interaction of sEVs in Fixed Recipient Cells

sEVs act as a signal mediator in different physiological and pathological conditions, such as inflammation, tumor development, tumor progression, metastasis, and regulation of immune responses.[1] When sEVs reach their destination, they fuse with the membrane of recipient cells and release their active and inactive cargo biomolecules in the cytoplasm to initiate a particular biological process. Normally, inactive EV cargo biomolecules are internalized efficiently through the endocytosis pathway.[118] The mechanism underlying sEV uptake by different recipient cells is yet unknown. Further, it is not necessary that all active EV cargo biomolecules have biological function only in the cytoplasm. Using confocal 3D microscopy, we have recently demonstrated the distribution of EV-DNA in different recipient cellular compartments. Interestingly, we have determined that nearly half of EV-DNA population stuck at the recipient cellular membrane whereas the remaining EV-DNA population overcame the cell membrane barrier and entered the cytoplasm and nucleus.[11]

As previously mentioned, super-resolution image is constructed from a large number of individual molecular localizations; therefore, the choice of blinking fluorophores becomes very important for SMLM. Only very few EV researchers have implemented SMLM on fixed cells for sEV-based functional studies. Chen et al., 2016 showed for the first time the interaction between breast cancer-derived exosomes (SKBR3) and human embryonic lung fibroblast (MRC-5) using PALM/STORM imaging.[47] In this study, they labeled the membrane of MRC-5 cells with PKH67 and CD63 (EV canonical marker) of SKBR3 sEVs with Alexa Fluor 647. In addition, they showed the colocalization of SKBR3 sEVs with lysosomes of MRC-5 cells (using LysoTrackerRed, Thermo Fisher Scientific) indicating that these sEVs are in the process of degradation. On the other hand, Zong et al., 2018. illustrated SMLM imaging of SKBR3 sEVs using the silicon quantum dots (Si QDs). They stained CD63 of SKBR3 sEVs using CD63 aptamers fused with Si QDs. The main advantages of Si QDs are high biocompatibility, low cytotoxicity and no requirement of special buffers for imaging. All these features make Si QDs an alternative and better fluorophore for SMLM, including live-cell imaging.[119]

Next, Lee et al. utilized SMLM to study the spatial distribution of genomic *Alu* elements around chromosome 9 centromeres. They employed combinatorial oligo fluorescence *in situ* hybridization (COMBO-FISH) probes that contain unique 15–25 nucleotides specific to these regions for labeling.[120] This labeling strategy can be applied to EV-DNA to study not only their spatial distribution in the recipient cells but also their arrangement with other DNA-binding proteins (e.g., histones).

4. Conclusion

Small EVs control many aspects of physiology and disease by delivering multiple bioactive molecules that modulate the behavior of recipient cells. However, the biology of sEVs biogenesis and uptake remained largely unexplored due to the lack of suitable technologies, especially for multicolor imaging. The size of sEVs falls below the diffraction limit resulting in highly blurred structures, when using the conventional light microscopy. In this regard, SMLM has gained particular interest for subdiffraction imaging of biological structures in both live and fixed cells. We discussed how SMLM techniques for cell imaging can be translated to the EV field to study sEV-to-recipient cell interactions at nanoscale level. As SMLM resolution goes to the molecular scales, choosing a labeling strategy that minimizes the linkage error is a crucial step for SMLM imaging of sEVs. This would allow the quantification of single proteins and nucleic acids at the "sub-vesicular" level, which would definitily help assesing the biology of sEV biogenesis and secretion. SMLM imaging of EV biogenesis will ultimately resolve sEV heterogeneity and define different sEVs subpopulations with special molecular signatures and, therefore, distinct functions in the recipient cells. SMLM technologies assisted with machine learning and artificial inteligence will help to gain further insights on sEV half-life and how they target different cell compartements, which would support their use as biomarkers and open new vistas in EV biology such as drugs formulation in sEVs.

SMLM field is continuously evolving and to fully exploit the resolution power of SMLM for EV biology, the 3D resolution live-cell and in vivo compatibility as well as the multicolor imaging require further improvement. EV biology is also a growing field of research and we believe that one day in the near future all sEV molecules will be individually characterized, localized, and tracked inside the recipient cells. To this end, much work needs to be done to make SMLM an easy-to-do technique for biologists in the EV field.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

extracellular vesicle (EV) imaging, exosomes, live-cell imaging, single molecule localization microscopy, small extracellular vesicles, super-resolution microscopy

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