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Rapid and accurate analysis of stem cell-derived extracellular vesicles with super resolution microscopy and live imaging

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ABSTRACT

Extracellular vesicles (EVs) have prevalent roles in cancer biology and regenerative medicine. Conventional techniques for characterising EVs including electron microscopy (EM), nanoparticle tracking analysis (NTA) and tuneable resistive pulse sensing (TRPS), have been reported to produce high variability in particle count (EM) and poor sensitivity in detecting EVs below 50 nm in size (NTA and TRPS), making accurate and unbiased EV analysis technically challenging. This study introduces direct stochastic optical reconstruction microscopy (d-STORM) as an efficient and reliable characterisation approach for stem cell-derived EVs. Using a photo-switchable lipid dye, d-STORM imaging enabled rapid detection of EVs down to 20–30 nm in size with higher sensitivity and lower variability compared to EM, NTA and TRPS techniques. Imaging of EV uptake by live stem cells in culture further confirmed the potential of this approach for downstream cell biology applications and for the analysis of vesicle-based cell-cell communication.

1. Introduction

Extracellular vesicles (EVs) are lipid membraned nanostructures secreted by cells either directly from the plasma membrane or via the endocytic pathway [1]. EVs contain and transport miRNAs [2], mRNAs [2] and active proteins [3] reported to modulate inter-cellular communication, with increased prevalence in a range of biological processes linked to cancer [4], neuroscience [5], and stem cell biology [6]. Stem cells have been reported to secrete paracrine factors largely via EVs, with relevance to immune modulation [7] and tissue repair [6]. In particular, mesenchymal stem cells (MSCs) are known to be a rich source of EVs suggested to promote healing in cutaneous wounds [8], bone fractures [9] and liver injury [10]. These observations indicate stem cells may provide a source of therapeutically useful EVs that could offer possible cell-free treatment strategies for regenerative therapy.

Cell-secreted EVs show a high degree of heterogeneity in size with apoptotic bodies and microvesicles ranging from 50 to 1000 nm, and exosomes ranging from 30 to 100 nm [1]. Exosomes can be separated into different size groups, with distinct mRNA and protein composition, and different effects on the gene expression of recipient cells [11]. It has recently been shown that EVs of 30 nm to 60 nm in size are more readily taken up by recipient cells within a 24-hour time period compared to larger EVs of 80 to 100 nm in size, resulting in higher motility of cells [12]. These recent reports highlight the importance of size as a differentiating factor for EV populations, underlining the analysis of particle size distribution (PSD) as a crucial parameter to characterise the structural and functional properties of EVs in cell biology.

Electron microscopy (EM), including scanning EM (SEM) and transmission EM (TEM), have emerged as standard techniques for EV characterisation, allowing high resolution imaging for the acquisition of size and morphology information [13], and immuno-labelling of samples to detect protein content [14,15]. Although the development of cryo-TEM has improved the preservation of sample structure and morphology [16], the uneven and inconsistent distribution of EVs onto EM grids makes it technically challenging to accurately measure concentrations. Two common alternatives to EM used to characterise EVs include nanoparticle tracking analysis (NTA) [17], and tuneable resistive pulse sensing (TRPS) [18]. However, the highly polydispersed

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nature of EVs make them challenging to measure using these techniques. For NTA and TRPS the minimal size range for EV detection is on average 70–150 nm, which excludes most exosomes [19]. The ability of NTA to accurately resolve two EVs, which depends on the light scattered by particles in Brownian motion [20], requires a 1.5 fold difference in their size [21], while its Raleigh approximation-based concentration estimation is strictly dependant on their refractive index, which varies with size and cargo [17,20]. By contrast, TRPS can provide more accurate size and concentration measurements by detecting current blockage from particles passing through a nanopore [22], but the detection of EVs < 100 nm is problematic due to nanopore blockage by larger EVs [23]. Therefore, these techniques have significant limitations including the need for several detection settings and calibration beads for NTA [20], and the application of multiple nanopore sizes to minimise blockage in TRPS [24]. Immunoblotting (e.g. western or dot blot) is generally performed alongside these analyses to confirm the presence of EVs based on their protein content. [14,25] However, the high heterogeneity in cargos exposes protein-based quantification of EVs to inaccuracy. For instance, CD63 has been observed to be inconsistently expressed in EVs isolated from different human prostate and breast cell lines [26], while other EV markers have been found to be unevenly enriched in different proportions depending on PSD [11]. This implies that in the absence of ubiquitous EV protein markers, immunodetection approaches are inaccurate and likely to misestimate the concentration of EVs present in cell samples.

Stochastic optical reconstruction microscopy (STORM) and direct-STORM (d-STORM) are emergent single-molecule super-resolution imaging techniques with a practical resolution limit of 20 nm [27]. STORM exploits the photoswitchable property of certain fluorescent probes to localise events with high precision, and reconstruct the acquired image at a high spatial resolution [28]. As a result, STORM has been used extensively to image and characterise subcellular structures with regards to their anatomy [29], organisation [30], and biomechanical [31] properties at the nanoscale. Recently, cancer cell-derived EVs labelled using AlexaFluor 647-conjugated anti-CD63 antibodies have been imaged at high resolution using STORM [32]. Since all EVs are lipid membraned structures [33], lipophilic dyes can provide a helpful alternative to label EVs [34], irrespective of the variability in their protein content. Dyes such as DiI and its derivatives exhibit photoswitching behaviour, shifting between brightly fluorescent (light) and dark states, which enables STORM imaging of lipid-based cellular structures including the plasma membrane and lysosomes [35]. Building on this, the present investigation sought to exploit d-STORM imaging using a variant of DiI to explore the possible labelling and direct characterisation of EVs released by stem cells as a powerful alternative to existing approaches for the study of EV trafficking.

2. Materials and methods

All materials were purchased from Thermofisher Scientific (UK) unless stated otherwise.

2.1. Cell culture

Mouse mesenchymal stem cells (MSCs, D1, ATCC CRL-12424) were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), 1% L-glutamine and 1% non-essential amino acids (NEAA). 0.25% trypsin-EDTA was used for splitting the cells. Primary mouse neural stem cells (NSCs) were isolated from adult mouse lateral ventricle tissue as previously described [36] and cultured in NSC medium (DMEM F12/Neurobasal (1:1) medium containing 0.5% (P/S) and 0.01% heparin, with B27 and N2 supplements, and bFGF and EGF (both 20 ng/μl)). Accutase (Sigma) was used to split NSCs.

2.2. EV isolation

Prior to EV isolation, MSCs were washed with Phosphate-buffered saline (PBS) and then incubated in with EV enrichment (Exo-E) medium - containing phenol red-free low-glucose DMEM,1% L-glutamine, 1% NEAA, 1% P/S and 10% Exo-free FBS (System Biosciences), with added DiD Vybrant Cell labelling solution according to manufacturer's instructions (5 μl/ml). After 6 h, the medium was collected and filtered using 0.45 μm syringe filters (SLS). For EV isolation, exoEasy Maxi Kit (Qiagen) was used according to manufacturer's instructions. For negative controls, freshly prepared [DiD in PBS], [DiD in Exo-E medium] and [DiD in serum-free Exo-E medium], were processed in the same way. Eluted EVs were either immediately diluted and used for size distribution and particle count analysis, or stored at −80 °C for use in cell culture experiments. Samples were sonicated prior to use using Bioruptor (Diagenode) at low power three times for 10 s.

2.3. TEM and cryo-TEM

For TEM, samples were prepared according to a published protocol with slight modifications [14]. Briefly, samples were fixed with 4% paraformaldehyde and added (5 μl/grid) to glow discharged (10 s at 5 mA using an Agar turbo coater aux power unit and dedicated glow discharge head) Formvar-carbon coated EM grids (EM resolutions), and adsorbed for 20 min. Sample grids were washed with PBS and incubated with 1% glutaraldehyde for 5 min, washed with sterile distilled water, and incubated with 3% uranyl-acetate for 15 min for negative staining. TEM was carried out using a Tecnai Biotwin-12 with an accelerating voltage of 100 kV. For cryo-TEM, glow discharged Holey carbon copper TEM grids were used (EM resolutions). Samples were let to adsorb onto the grids (5 μl/grid) for 20 min before the excess solution was removed using filter paper and the grids allowed drying under ambient conditions. Samples were then frozen using a Gatan CP3 plunge freezing unit, blotting for 1 s and freezing in liquid ethane. Samples were transferred to cryo-TEM storage boxes and then loaded into a Gatan 626 cryo-TEM holder on a JEOL 2100+ TEM. Images were acquired for 2–4 s at a dose of below 10 e/A^2, using a US1000 CCD camera and Digital Micrograph GMS 3.

2.4. Dot blot immunodetection

MSCs and MSC-derived EV extracts were lysed using RIPA lysis and extraction buffer with added proteinase inhibitor and phosphatase inhibitor cocktails (Sigma). Protein concentration was measured using a Bradford assay (Sigma). For dot blot analysis [37], 10 μg of protein from each sample were added onto nitrocellulose membranes and dried for 10 min. The membranes were blocked using 1% skimmed milk in TBS-T (0.1% Tween20 (Sigma) in Tris buffer) and incubated with primary antibodies against CD63, TSG101, or GM130 (Santa Cruz) for 30 min. Membranes were then washed with TBS-T, incubated with peroxidase conjugated secondary antibody (vector laboratories) for 30 min, and then washed with TBS-T, before a 1-minute incubation with ECL detection reagent. Membranes were immediately imaged using LAS-4000 (Fujifilm).

2.5. d-STORM characterisation

For d-STORM imaging, a 1 in 1000 dilution of DiD-labelled EVs in PBS was seeded onto poly-L-lysine (Sigma) coated 4-well glass bottom Petri dishes (Greiner-Bio). Imaging was performed using a Zeiss Elyra PS1 super resolution microscope, with an α-Plan Apo 100×/1.46 oil immersion objective in TIRF (Total internal reflection microscopy) mode. Before imaging, 30 °C oil (Zeiss, Immersol™ 518F/30°) droplet was placed on 100× objectives. The LP 650filter was used to visualise EVs. TIRF was used to visualise and scan the EVs bound to the cover glass, automatic focusing (definite focus) maintained the desired focal