Single particle profiler for measuring properties of nano-sized bioparticles

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Abstract

It is technically challenging to study properties of nanoscale bioparticles such as lipoproteins, extracellular vesicles (EVs) and viruses in a high-throughput manner. We developed a high-throughput analysis method, called single particle profiler (SPP), based on the fluorescence signal from thousands of individual particles diffusing through a ≈0.5 femtoliter observation volume. This method provides information on surface properties, biophysical properties and diffusion of particles, simultaneously. We applied SPP to study properties of liposomes, lipid nanoparticles, lipoproteins, viruses and EVs.

Main text

Physiological nanometer-sized particles in human body are of utmost importance for health and disease. For example, lipoproteins (5 – 60 nm) transport lipids to maintain cellular metabolism[1]; extracellular vesicles (EVs) (<200 nm) take part in immune response, cell-cell-communication and signaling[2]; and finally viruses with an average size of 100-200 nm cause variety of diseases. Moreover, liposomes and lipid nanoparticles are widely employed for drug delivery and vaccines[3]. The analysis of their surface and biophysical properties can shed light on their structure, functions, and behavior in health and disease.

Most of the existing methods to study bioparticles rely on biochemical analysis, mass spectroscopy, TIRF microscopy and conventional flow cytometry. Biochemical methods and mass spectroscopy are bulk methods (i.e., lacks single particle sensitivity). TIRF microscopy provides excellent signal to noise in truly single particle manner; however, it requires fixation of the vesicles on the surface and yields low-throughput data. Flow cytometry, on the other hand, is a high throughput method; however, it is generally suitable for cell-sized objects. Recently, there have been attempts to analyze small EVs with flow cytometry[4–8] and extract meaningful information from background reference noise[9]. Moreover, there is an ongoing development of the "nano-flow" devices that rely on microfluidic equipment[10,11]. However, these methodologies are still limited to particle size of >200 nm and require dedicated and often expensive equipment.

We designed a method, single particle profiler (SPP), that is based on fluorescence fluctuations of thousands of diffusing particles in solution on a commercially available confocal microscopy (Figure 1a). Briefly, fluorescently labeled particles diffuse through the diffraction-limited observation volume (≈0.5 fL) where the fluorescence emission from multiple channels is monitored continuously (as in fluorescence cross correlation spectroscopy[12,13]). Next, we identify individual peaks in intensity fluctuations in multiple channels using the custom, freely available python script (Figure 1b, see Supporting Movie 1 for the tutorial of the software and Github for downloading the software). Based on the intensity of each individual peak in multi-channels, we can construct a density plot (Figure 1c) where every dot represents the fluorescence intensities in two separate channels, therefore, like flow cytometry, this approach can be used to measure the fluorescence intensities in single nanometer-sized particles smaller than 250 nm. Such information can be used for *surface profiling* and *biophysical profiling* of particles such as lipoproteins, viruses and EVs.

First, to demonstrate the ability of SPP for *surface profiling*, we prepared liposomes loaded with either fluorescently-labelled cholesterol (Topfluor cholesterol or TF-Chol) or fluorescently-labelled phospholipid (Abberior Star Red phosphatidylethanolamine or ASR-PE) or both. TF-Chol liposomes showed high green signal but no red signal, ASR-PE liposomes showed high red signal but no green signal and TF-Chol/ASR-PE liposomes showed both green and red signal (Figure 1c) confirming the applicability of our methodology for surface profiling. To further demonstrate the strength of SPP, we changed the ratio of green and red signal in liposomes. SPP could capture the small changes in intensity ratios of red and green (Figure 1d).

Besides surface profiling using fluorescent lipids or proteins, biophysical properties in nanosized bioparticles can also be studied with SPP using ratiometric environmental sensitive probes (Figure 1e). To demonstrate the ability of SPP for *biophysical profiling*, we prepared liposomes of distinct lipid compositions and supplemented them with 0.1 mol % of ratiometric dye Nile Red 12 S (NR12S, Supplementary Figure 1). The emission spectrum of this dye is sensitive to fluidity of the lipid environment which is measured with an index called Generalized Polarization (GP). Numerical value of GP changes between +1 and -1 and is inversely proportional to membrane fluidity (e.g., higher GP = lower fluidity). NR12S-labelled liposomes were excited with 488 nm laser and the fluorescence intensity in green and red parts of the spectrum was recorded simultaneously in two separate channels. Then, peaks were called in both channels and GP was calculated for each peak. We prepared liposomes of different membrane fluidity by using saturated lipids, unsaturated lipids (18:1/18:1 DOPC, 18:1/16:0 POPC, 16:0/16:0 DPPC) and cholesterol in various combinations. As expected, membrane fluidity increased with varying cholesterol and lipid saturation (Figure 1e,f, Supplementary Figure 2, 3).

One of the unique advantages of SPP compared to cytometry tools is the ability to measure diffusion of particles since it is based on fluorescence fluctuations. Diffusion of particles (directly related to particle size) can be an additional parameter to separate different particles. To demonstrate this, we quantified the diffusion coefficient of liposomes and used this parameter in combination with membrane fluidity to perform clustering analysis (diffusion vs GP). Data that represented distinct lipid compositions organized in well-resolved clusters (Figure 1g). Another advantage of SPP compared to bulk techniques is single particle readings from thousands of particles, which allows us to dissect multicomponent mixtures. To show this, we prepared a mixture consisted of POPC liposomes and DPPC/chol liposomes. Analysis of such mixture indeed revealed presence of two liposome populations: one population of low order (POPC liposomes) and a second population of high order (DPPC/chol liposomes) (Figure 1h). The sensitivity of such separation was further tested by preparation of liposomes with different percentages of cholesterol, clearly showing broadening of GP histograms and heterogeneity of the particles even when the compositions of mixed liposomes are fairly similar (Figure 1i, Supplementary Figure 4). Importantly, single particle capability of SPP allows us to perform advanced statistical analysis of GP data and histograms. For example, standard deviation of GP data as well as sigma (σ) of GP histogram provide information on the heterogeneity of the sample. This was demonstrated for binary mixtures of POPC and POPC/chol liposomes (Figure 1i). Changing the amount of cholesterol in the POPC/chol

component of the liposome mixture, we demonstrated that, both standard deviation of GP and sigma increased, suggesting more heterogenous liposome distribution compared to pure POPC liposomes (Figure 1j, k).

In summary, liposomes provided excellent validation for the performance of SPP. We showed that *(i)* surface and biophysical properties of nanosized liposomes can be studied with SPP in single-particle and high-throughput manner; *(ii)* multiple parameters (such as diffusion and fluidity) can be obtained for clustering analysis; and *(iii)* sample heterogeneity can be studied using statistical analysis. Next, we applied this method for profiling physiological particles such as lipoproteins, virus-like particles, EVs and lipid nanoparticles.

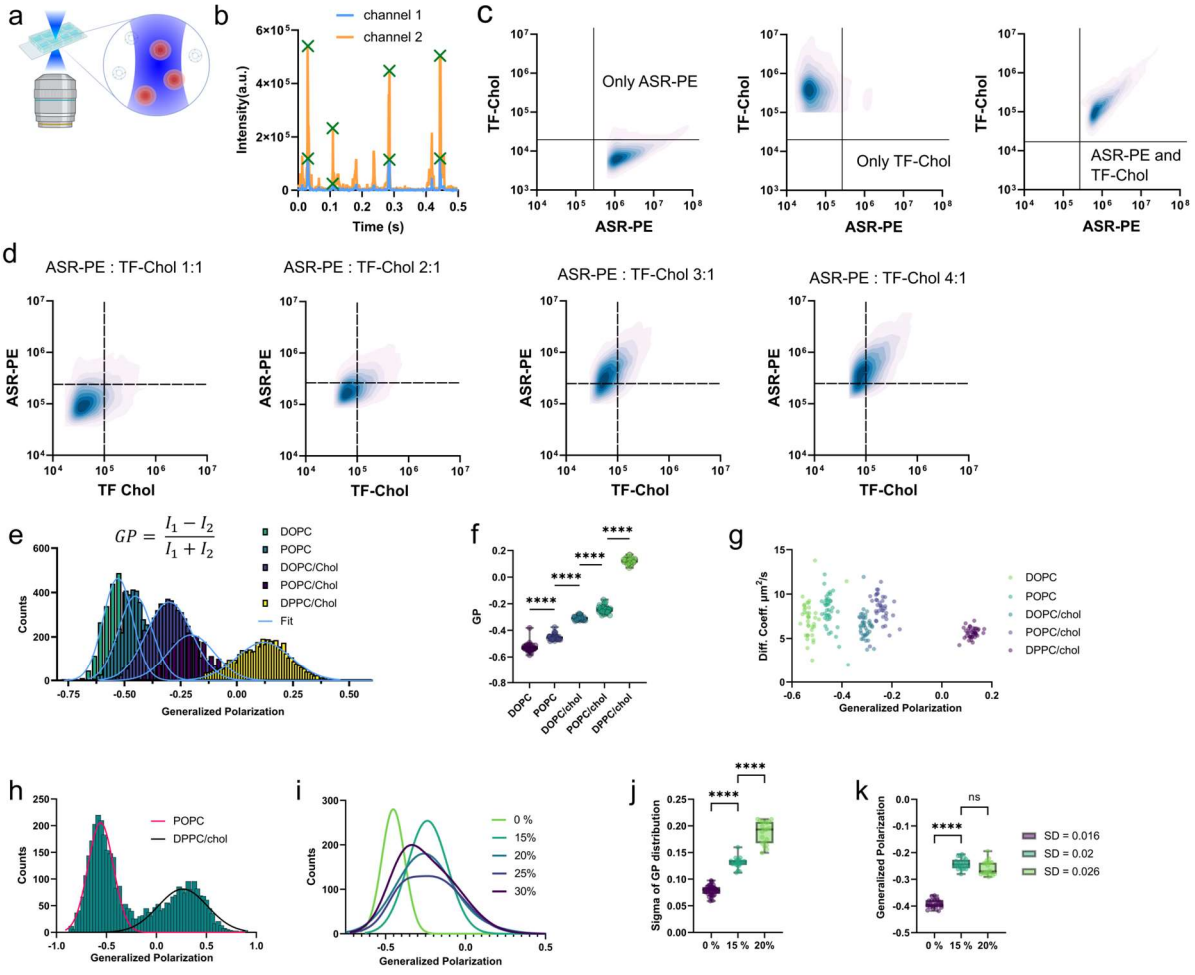

Figure 1| Single Particle Profiling. a) SPP setup; **b)** Fluorescence intensity traces and peak calling; **c)** 2D density map of fluorescence intensities for liposomes loaded with ASR-PE and TF-Chol: density plot from liposomes loaded with both dyes belongs to the 3rd quadrant of the plot, whereas the density plots for liposomes loaded with single dyes are located in second and fourth; **d)** 2D density plots for liposomes loaded with TF-Chol and ASR-PE at different ratios; **e)** Ratiometric calculation of generalized polarization for each peak and the histograms of generalized polarization for liposomes of different lipid compositions; **f)** Mean values of gaussian fitting of histogram as in panel e from multiple repetitions; **g)** Diffusion coefficient vs. GP dot plot for liposomes; **h)** The GP histogram of the mixture of liposomes with distinct composition shows two distinct populations that can be attributed to the components of the mixture; **i-k)** Data for binary mixture of pure POPC liposomes and POPC/chol liposomes with different percentage of cholesterol. i) GP histograms show one population for 0% chol – 20% chol and two distinct populations starting from 25% chol. Even where multiple populations cannot be resolved at low cholesterol mixtures, heterogeneity manifests in broadening of the distribution (j) and increase in mean and standard deviation of GP (k).

It is generally assumed that lipoproteins are similar in physical properties regardless of health conditions; therefore, current diagnosis tools for diseases such as hypercholesterolemia solely rely on lipoprotein counts in blood. However, lipoprotein biophysical properties also play a role in lipoprotein function[14]. To measure the fluidity of lipoproteins, a critical factor for their lipid delivery[15], we extracted lipoproteins from blood plasma of healthy individuals (three donors) as well as from the individuals with diagnosed obesity (five donors). From each plasma sample we isolated three major types of lipoproteins: high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL). The sizes of HDL and LDL appeared similar for all individuals (Supplementary Figures 5,6). We labelled all LPs with NR12S and performed biophysical profiling. First, the diffusion data confirmed that HDL particles are the smallest and VLDLs are the biggest for all donors (Figure 2a, b). Interestingly, for all donors, LDLs demonstrated the lowest membrane fluidity. Notably, there is an obvious difference in membrane fluidity for the same type of LPs extracted from healthy vs obese donors. To illustrate this distinction, we plotted clusters of diffusion coefficient vs GP for all lipoproteins and observed a clear segregation of the same type of lipoproteins from healthy vs obese individuals (Figure 2e). Particularly pronounced is the difference for VLDL and LDL, which are both more fluid in obese individuals. This highlights the importance of the "biophysical quality" of lipoproteins in health and disease that can potentially pave the way for novel diagnostic tools as well as disease definition in general.

There is growing evidence that biophysical properties of viruses are key for their budding as well as their interaction with their host[16,17]. To study such properties of viruses, we produced SARS-CoV-2 (beta and delta variants) and Ebola virus-like particles (VLPs). We labelled these VLPs with NR12S and performed SPP. Interestingly, the membrane fluidity of Ebola VLPs was the lowest, while there was only a minor difference between VLPs with SARS-COV-2 spike variants (Figure 2f, Supplementary Figure 7). This shows that different viruses gather different membranes from the host while budding. Biophysical properties of EVs are also important for their function[18]. Depending on the metabolic or immunological status of cells, lipid and protein content as well as surface signature of the resulting EVs may vary[6]. Since the knowledge about EV content is crucial to determine their heterogeneity, to understand the molecular content and related functions and to reveal their therapeutic effects, we tested whether SPP would be applicable to study EVs. To address the question of heterogeneity of EVs from different sources, we measured EVs isolated from different cell lines. There was a clear difference in EV fluidity between different cell lines (Figure 2g, Supplementary Figure 8). Importantly, GP standard deviation and sigma parameters showed the polydispersity of the EVs in terms of biophysical properties (despite seemingly monodisperse size distribution, Supplementary Figure 1) which will have a great potential for the EV community as it provides an additional quality control for EV preparations[19].

Lipid nanoparticles are widely used as drug delivery and vaccine agents. Their composition, organization and biophysical properties are crucial for efficient uptake by target cells. As a proof-of-principle experiment, we incorporated NR12S into LNPs of varying cholesterol content and observed that increasing cholesterol amount decreases fluidity of LNPs (Figure 2h, Supplementary Figure 9). SPP opens a new window to study the biophysical properties of

different LNP formulations and the standard deviation of GP and sigma can be utilized to probe the LNPs heterogeneity.

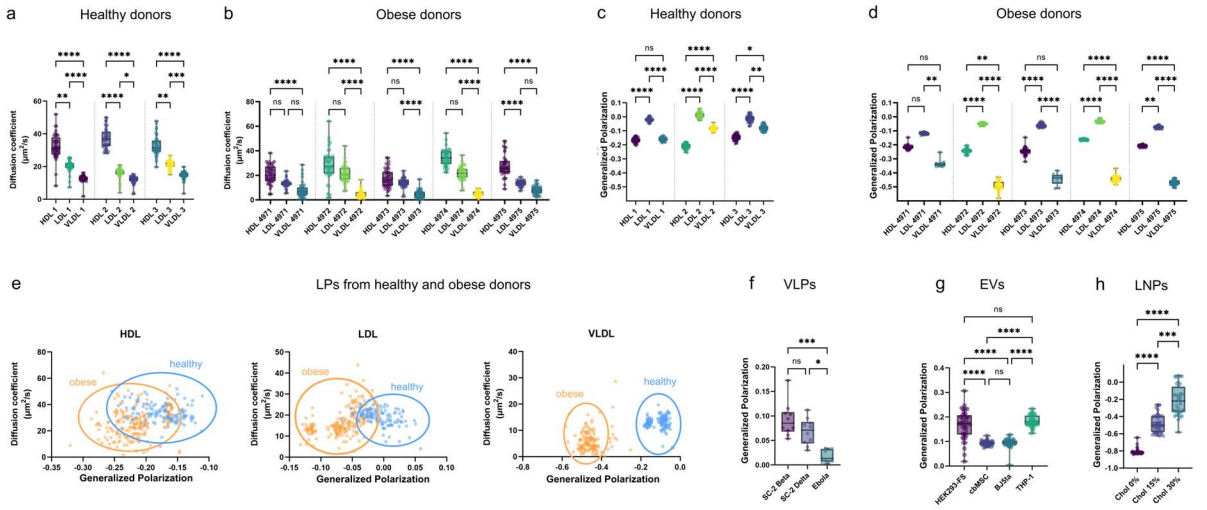

Figure 2| Nanometre-sized lipid particles profiled by SPP. Diffusion coefficients of LPs isolated from blood plasma of apparently healthy (**a**) or obese (**b**) individuals; GP of LPs isolated from blood plasma of apparently healthy (**c**) or obese (**d**) individuals; **e)** Dot plot of diffusion coefficient vs GP for healthy and obese individuals. Membrane fluidity measurements of **f)** virus-like particles, **g)** EVs and **h)** Lipid nanoparticles.

Here, we presented and validated a new analysis method for high throughput single particle profiling. This method is based on commercially available and highly accessible confocal systems and has wide applicability for resolving content and organization of the nanosized physiological particles. SPP does not require dedicated equipment and is not limited to fixed spectral regions thanks to the available detector flexibility in confocal systems. SPP provides information on lipid and protein content, biophysical parameters and polydispersity of nano-sized bioparticles, which can shed light on the role of these particles in health and diseases such as in metabolic disorders, cancer and host-pathogen interaction.

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