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Exosome and Microvesicle Characterization by Nanoparticle Tracking Analysis

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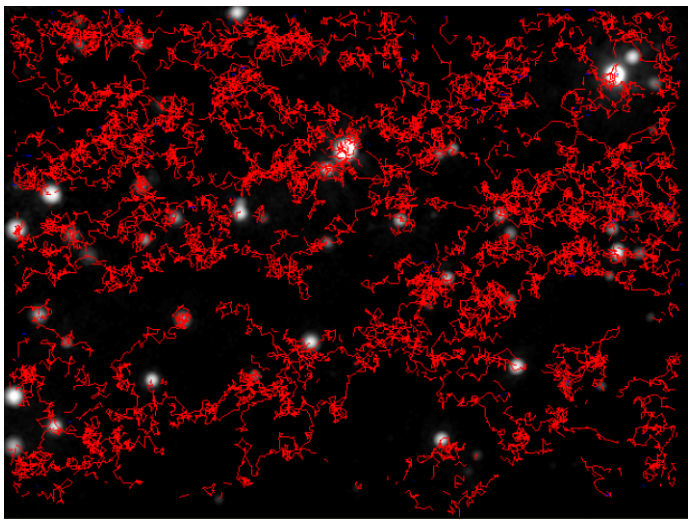
In this white paper, NanoSight's applications team describes the operation and application of NTA to address the characterization challenges of exosomes and microvesicles.

NanoSight is a company that develops, manufactures and supplies nanoparticle characterization instrumentation. The sizing and counting of nanoparticles in liquids is vital in many areas. NanoSight enables the user of its systems to directly visualize nanoparticles particle by particle to derive individual numbers rather than producing the average numbers as may be seen by other techniques, e.g. Dynamic Light Scattering (DLS).



NanoSight's NTA software showing exosomes and microvesicles being tracked whilst moving under their Brownian motion in liquid

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Exosome and Microvesicle Characterization by Nanoparticle Tracking Analysis

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Introduction

Exosomes and microvesicles are small vesicles shed by cells, which play an integral role in intercellular communication. Whilst broadly accepted definitions perhaps require refinement, an exosome can be broadly defined as a 40-100nm diameter membrane vesicle of endocytic origin released by most cells upon fusion of the multivesicular bodies with the plasma membrane – presumably as a vehicle for intercellular communication [1]. Microvesicles are generally classed as 100nm – 1µm vesicles which directly bud from the plasma membrane. It is their role as potential biomarkers which makes this field of research so exciting. Taylor *et al* [2] describe how the presence of tissue/cell type-specific marker proteins associated with a specific protein can be used to determine the originating cell. In lay terms, if one can understand the message being transported by these vesicles and understand where that message is originating from, one can potentially develop a diagnostic test which can detect and perhaps predict the onset of cardiac disease for example. Furthermore, it is their ubiquitous presence in a broad range of biological and physiological processes as well as their elevated levels in blood associated with many diseases such as cancer, cardiac disease and pre-eclampsia which opens avenues for the development of exosome/microvesicle based diagnostics across a broad range of conditions.

It is becoming increasingly apparent that the techniques traditionally used to both isolate and characterise biological materials are either unsuitable or have not been developed and refined for work with exosomes and microvesicles. As a result, the rate of development and discovery is hampered in what is potentially a broadly significant area of research. Van der Pol *et al* [3] provide a nice summary of the techniques currently available for the study of exosomes and microvesicles. Furthermore, Dragovic *et al* [4] complement the major conclusions of the Van der Pol review and suggest that whilst electron microscopy can demonstrate the presence of microvesicles and exosomes, it is not quantitative and requires extensive and often intrusive sample preparation. They go on discuss how ELISAs have issues with the inability to capture all of the particles present, as well as being influenced by soluble antigens. Flow cytometers would be deemed as perhaps the 'go-to' instruments of choice but particles smaller than 300nm cannot be detected by this technique (lower limits of detection are open to some argument and newer instruments continually push the lower limit of detection). Both of these groups discuss the potential of Nanoparticle Tracking Analysis (NTA), and perhaps more importantly, Fluorescent Nanoparticle Tracking Analysis (FNTA) as a potentially well suited technology for the measurement of these particles.

What Is Nanoparticle Tracking Analysis?

Nanoparticle Tracking Analysis is a technique which visualises the light which is scattered from an exosome or microvesicle live and direct in liquid suspension. A finely focused laser beam is passed through an optical interface such that it refracts through a thin layer of sample in liquid suspension. Light is scattered by the exosome/microvesicle in the path of the laser beam and is collected at 90^{o}

using a light microscope, and then imaged using a Scientific Complementary Metal-Oxide Semiconductor (Scientific CMOS) camera which is mounted on the microscope (see Figs 1.1 and 1.2).

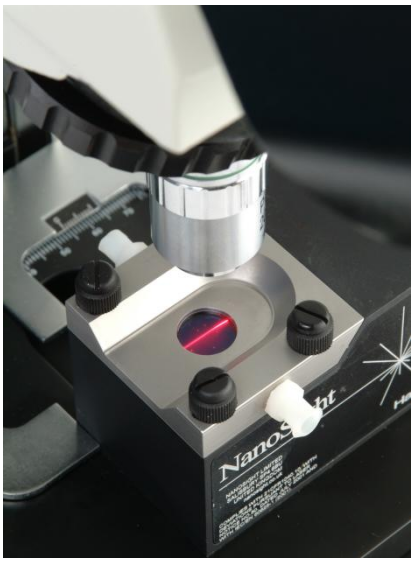


Fig 1.1 Image of Sample Cell

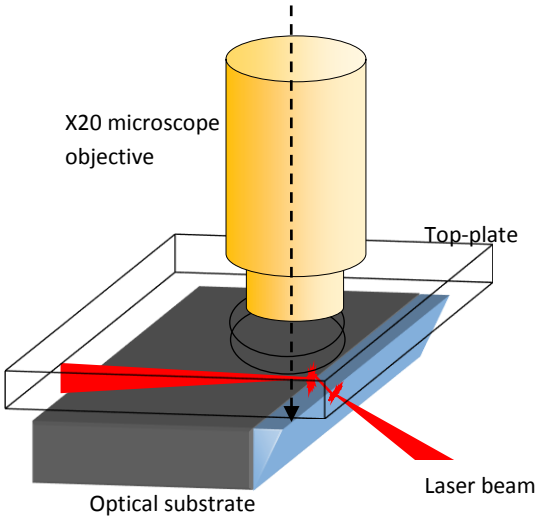


Fig 1.2 Schematic Showing Optical Configuration

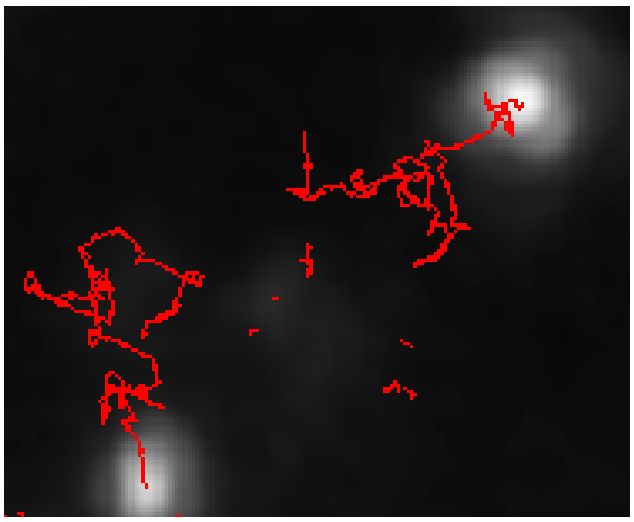
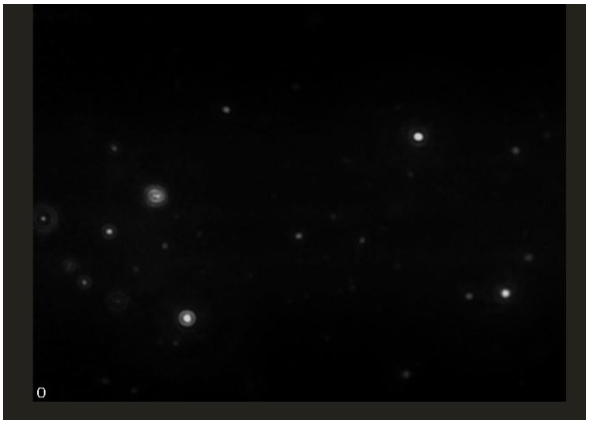


Fig 2. An enlarged image of typical tracks of particles moving under Brownian motion. Note: The image is from the scattered light and is not a resolved image of the particle. Therefore the technique does not provide structural information.

Particles move in liquid under Brownian motion, and are visualised by the technology. The NTA software uses image analysis to find the centre of each particle and then, over the course of the video, the particle is tracked and its diffusion coefficient measured (see Fig 2). The Stokes Einstein equation (see Eq 1) is used to relate the measured diffusion coefficient to particle size, on a particle-by-particle basis, offering high resolution distributions capable of detecting subtle sub-populations. The fact that particle size is derived on a particle-by-particle basis overcomes the inherent intensity bias associated with ensemble techniques such as Dynamic Light Scattering (DLS), which produce average particle sizes biased towards the larger, brighter microvesicles within a sample.

$$\frac{\overline{(x,y)}^2}{4} = Dt = \frac{TK_B}{3\pi\eta d} \qquad \text{Eq 1}$$



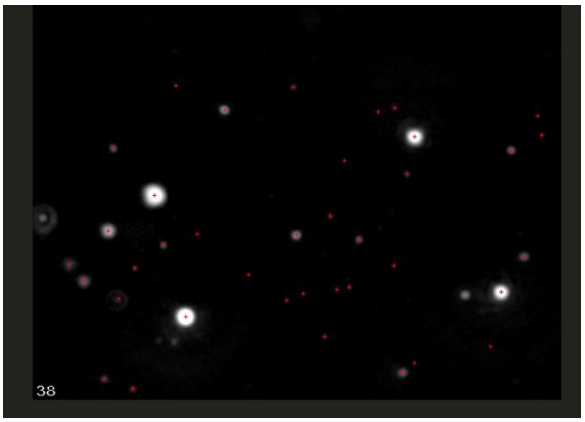
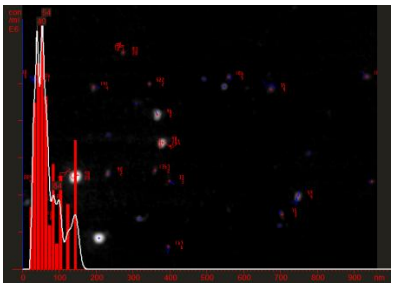
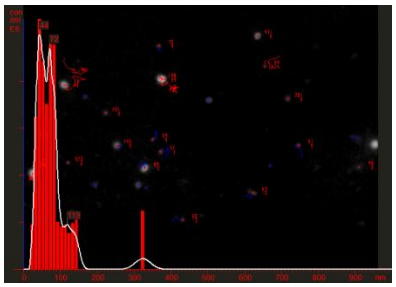


Fig 3.1 Image of exosomes as viewed by NTA. *Fig 3.2 Exosomes identified prior to sizing by NTA*

Fig 3.1 shows a still frame from the video captured by the technique; 3.2 shows how the NTA software has identified the particles; the Brownian motion of these particles is then tracked and used to calculate particle size as previously described. Fig 2 shows the tracking of each particle with the red trace denoting the Brownian walk that each particle travelled over the course of the analysis.





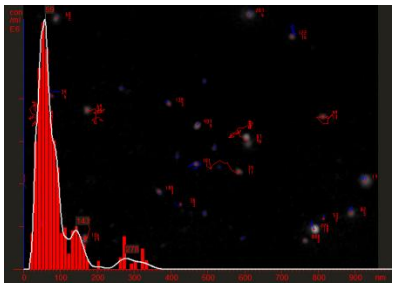


Fig 4.1 *Fig 4.2* *Fig 4.3*

Figs 4.1 – 4.3, show the process of how the exosomes are tracked and a high resolution size distribution measured. Fig 4.1 shows the first few seconds of tracking, as more data is captured the distribution starts to smooth and take shape a stable distribution of particles is shown in Fig 4.3. This process generally takes 30-60 seconds.

Possibly more importantly than its ability to measure particle size, the technique can also measure the concentration of particles within a preparation and can plot the number of particles within a given size class – expressed in terms of particles per ml. Figs 4.1-4.3 show a number versus size distribution with the Y axis showing particle concentration (in terms of particles $x10^6$ per ml)

The technique can image the fluorescent signal (FNTA) from an appropriately labelled vesicle, with a range of excitation wavelengths possible. Furthermore, particle size and concentration can be measured simultaneously whilst operating in fluorescence mode. So it would appear that FNTA provides the researcher with the measurands required to study the nuances present in clinical samples. Changes in the size of exosome/microvesicles, changes in the concentration of particles or changes in the distribution of particle sizes can be measured and potentially linked to the onset of disease. Finally, through fluorescence, FNTA provides the ability to discriminate target exosomes/microvesicles associated with a specific disease or cellular origin, from the host of

naturally occurring exosomes/microvesicles which are present due to the naturally occurring physiological processes within the body. In addition, the ability to work in fluorescence mode potentially by-passes the requirement to purify clinical samples (by ultracentrifugation/size exclusion chromatography/chromatography etc) and avoids the potential difficulties and variables presented by the method of purification itself. Tauro *et al* [5] have recently published a study looking at the differences in exosomal preparations produced by ultracentrifugation, density gradient centrifugation and immunoisolation using antibody labelled magnetic beads. The efficiency of the purification technique was monitored using electron microscopy as well as enrichment in exosomal markers as measured by Western Blot. The study demonstrated that whilst all the purification techniques produced exosome sized particles which were positive for exosomal markers, there were significant differences in the amount of positives. The immunoisolation method showed at least two times more positives than either the ultracentrifugation or density gradient centrifugation. The experiment proves that inconsistencies in purification techniques exist. As a result, it would be beneficial to by-pass them, or at the very least, the experiment demonstrates the need for standardisation of purification techniques if slight but perhaps clinically significant differences are to be discovered.

Case Examples – Use of NTA for the Characterisation of Exosomes and Microvesicles:

NTA was first assessed as a method for the analysis of exosomes and microvesicles by research groups working in the Departments of Haematology & Thrombosis and Reproductive Biology at the University of Oxford, England.

Harrison (2008 and 2009 and Harrison *et al* 2009) [6,7,8] were primarily interested in identifying new methods by which detection limits of >300nm for the popular and widespread technique of flow cytometry could be improved, given the proportion of microparticles below this limit was at that time unknown. They assessed a conventional DLS instrument and NTA and showed that while both systems gave similar results on calibration quality beads over the size range 50–650nm, measurement of purified microparticles (MPs) by NTA gave a polydisperse distribution up to 1000nm but with a predominant population in the 50nm to 300nm range. Analysis of diluted platelet free plasma (PFP) in PBS (1:40–1:160) suggested that the concentration of particles was 200–260×10^9/L which was 1000 fold greater than previous estimates. They concluded that while both techniques were rapid and capable of measuring over the entire size range of MP sizes to be expected in biological fluids, NTA exhibited superior resolving power in broad distributions.

Sheldon *et al* (2010) [9], in their study on notch signalling to endothelium at a distance by Delta-like 4 incorporation into exosomes, used NTA to confirm that their exosomes were only slightly larger than the suggested size of exosomes (modal size of 114nm for HUVECs and 120nm for U87 cells, compared with published sizes of 50-100nm). They stated that sizing of exosomes by electron microscopy was subjective and limited in that underestimation of size resulted from fixing and dehydration. NTA on the other hand allowed an objective and more accurate estimation of size of exosomes in a buffer such as PBS.

Dragovic *et al* [10] have most recently used both flow cytometry and NTA to rapidly size, quantitate and phenotype cellular vesicles. Their interest was in the study of cellular microvesicles (100nm-1um) and nanovesicles (< 100nm; exosomes) isolated from the placenta. These particles could have a major role as novel biomarkers for the condition of pre-eclampsia. Dragovic and her co-workers

used a commercially available flow cytometer (BD LSRI) employing side-scatter threshold, and showed that they could analyse microvesicles ≥ 290nm but nothing smaller. However, they demonstrated that NTA could measure cellular vesicles down to approximately 50nm.

Furthermore, using a human placental vesicle preparation in combination with a fluorophore labelled anti-placental alkaline phosphatase antibody (NDOG2-Qdot605), flow cytometry showed that 93.5% of the vesicles labelled positive for NDOG2 with over 90% of the vesicles being below 1000nm in diameter, the main population being between 300-400nm in diameter (Dragovic *et al* 2011b). However, when the same sample was studied by fluorescence NTA, the results showed a size distribution of NDOG2-labelled vesicles ranging from 50-600nm, with peaks at 100nm and 180nm. Analysis of total cellular vesicles in ultracentrifuge pellets of platelet free plasma (n=10) revealed that ~200 fold more vesicles were detectable using NTA (mean vesicle size 251±35nm) vs. flow cytometry. They concluded that these results demonstrate that NTA is more sensitive than conventional flow cytometry and greatly extended their capabilities for the analysis of microvesicles and nanovesicles (Dragovic *et al*) [4].

In a further extension to their work the Oxford group, Alvarez-Erviti *et al* (2011) [11] used NTA to show that exosomes played a role in the transmission of alpha-synuclein, aggregation of which is known to be important in Parkinson's disease pathology. These mechanisms they elucidated were considered to potentially provide a suitable target for therapeutic intervention.

Knowing that flow cytometry detects only a fraction of cell-derived microvesicles and nanovesicles in plasma (PMV), Gardiner *et al* (2011) [12] recently exploited the sensitivity of Nanoparticle Tracking Analysis and showed NTA sizing is not dependent on the refractive index of the exosomes, whereas sizing of exosomes by flow cytometry requires suitable calibration. Furthermore, FNTA analysis of PMV, achieved by labelling with a quantum dot-conjugated cell-tracker peptide, produced vesicle counts of $1.49x10^7$/µL for PFP and $1.20x10^7$/µL for the reconstituted pellet. >95% of all pelleted particles labelled with the cell tracker, compared to $<0.1x10^7$/µL (<0.02%) of the vesicles in the supernatant. The latter was stained with a lipophilic dye, indicating that these were probably lipoprotein vesicles which have a similar size profile to PMV and low density. This suggests that PFP comprises a large population of low density vesicles that are not cellular derived. The presence of lipoproteins will become problematic for flow cytometry as particle size detection limits continue to fall. The mean PMV (pelleted) count was 1.82x107/µL (SD 0.78), with a mean modal size of 92.7nm (SD 6.9nm) and a mean median size of 107.3nm (SD 9.8). The size distribution showed that the 75% of PMV were <150nm, while <2% were greater than 300nm; the minimum size detection limit of conventional flow cytometers. Pointing out that even the new ultra-sensitive flow cytometers only detect between 10,000 and 40,000 PMV/µl, Gardiner concluded that NTA detects approximately 100 times more PMV than the most sensitive flow-cytometers.

Gercel-Taylor *et al* (2012) [13] recently demonstrated a link between the concentration and size of circulating vesicles between patients with benign ovarian disease and ovarian cancer, with a x4 increase in the concentration of vesicles measured by NTA. The study went on to demonstrate how quantum dots conjugated with antibodies for CD63 (exosome marker) or EpCAM (which is a marker of vesicles derived from epithelial tumours) were used to phenotype the collected exosomes.

Conclusion:

There is a wide range of papers demonstrating the use of NTA and FNTA in the study of clinically derived exosomes and microvesicles. The technique seems to provide the measurands required to study the subtle nuances between samples in this broad ranging area of research. The technique measures high resolution size distributions, allowing sub-populations of vesicles to be easily identified. The technique can measure the concentration of vesicles within each size class with numerous papers showing a link between concentration of vesicles and disease. Finally the technique can work in fluorescence mode and as such allow the discrimination and measurement of appropriately labelled target vesicles. The technique provides a real alternative to commonly used flow cytometric technologies which are limited by their lower limit of detection.

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