ONE BY ONE

Measuring nanoparticles becomes increasingly important in healthcare, pharmacology, and environmental sciences, but measuring and obtaining relevant information from single nanoparticles requires cutting-edge technology. Therefore, we are developing a metrological flow cytometer that will be capable to traceably measure the concentration, size, and refractive index of single nanoparticles directly in suspension. This new instrument will have multiple applications not only for the detection of extracellular vesicles, but also for the determination of nanoplastics or the calibration of reference materials.

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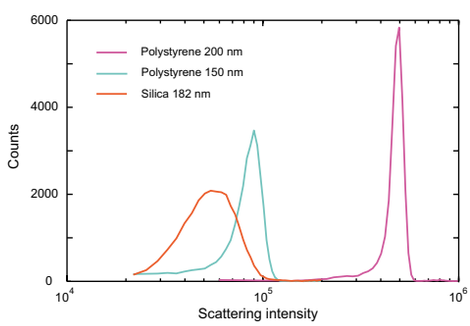
Introduction

Nanoparticles are receiving an increased interest for use in agriculture [1], environmental sciences [2, 3], healthcare [4] and pharmacology [5]. Therefore, traceable measurements of nanoparticles are becoming increasingly important. When measurements are traceable, they can be linked to the International System of Units (SI) by (inter)national measurement standards.
Current techniques might be able to measure the size of nanoparticles or the refractive index of bulk material, but what they lack is the ability to measure the refractive index of nanoparticles, or to do traceable measurements in fluid. Presently, the refractive index of single nanoparticles can be neither traceably nor untraceably measured. Traceable refractive index measurements of nanoparticles are necessary, since the bulk refractive index of a material can differ from the refractive index of nanoparticles (either in solution or as a collection of individual particles) of that same material [6].

The refractive index is a material property and can be used to distinguish between materials, or to determine which material it concerns. Without knowledge of the refractive index, nanoparticle sizes cannot be traceably measured in light-based detection methods such as flow cytometry.



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Scattering of polystyrene and silica nanoparticles. While 200-nm and 150-nm polystyrene particles can be distinguished, 150-nm polystyrene and 182-nm silica particles are indistinguishable despite their refractive index difference.

Metrological flow cytometer

Figure 1 shows the light-scattering intensities of three different particles measured using flow cytometry. The light scattering signal of particles depends on their size and refractive index. We can see that polystyrene particles of 150 nm and 200 nm can be distinguished, but that 150-nm polystyrene and 182-nm silica particles cannot be distinguished. Thus, without knowledge of both the size and refractive index of these particles, flow cytometry cannot distinguish between similar scattering signals. While particle sizes can be measured with techniques such as atomic force microscopy, the refractive index of nanoparticles cannot yet be traceably measured.

Because there is no technique able to measure the refractive index of particles in fluid, we developed a measuring set-up at the Dutch National Metrology Institute VSL in collaboration with Amsterdam UMC, as part of the METVES II project. The set-up, called the metrological flow cytometer, can be used to traceably determine both size and refractive index as well as number concentration of nanoparticles.

Principle of flow cytometry

Flow cytometry is a light-based detection method that detects microparticles and nanoparticles in fluids. Currently, commercial flow cytometry is mostly used to measure the cells present in blood plasma, but flow cytometry has the potential to measure nanoparticles present in all body fluids [4, 7].

Figure 2a shows the principle of commercial flow cytometry. In flow cytometry, the sample flow, which contains the particles that we want to measure, has a lower flow rate than the sheath flow. The flow rate difference results in hydrodynamically focussing of the sample flow into a narrow stream in the centre of the flow cell. Ideally, the particles in the sample flow pass the laser one by one to avoid swarm

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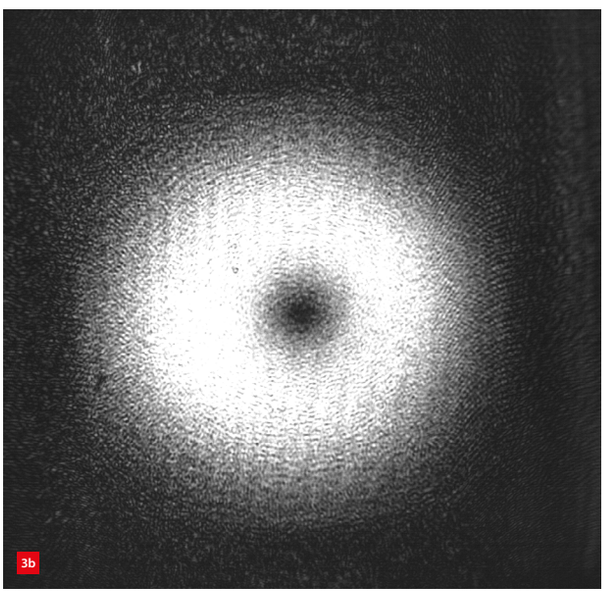
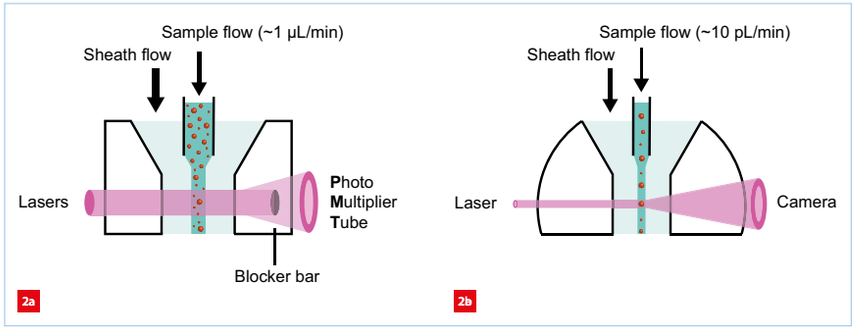
detection. When the particles are illuminated by the laser beam, light is scattered by the particles into all directions. The scattering signal is measured in forward scattering and sideward scattering directions on the detectors. The detectors of commercial flow cytometers are either photomultiplier tubes or photodiodes. The measured scattering signal of the particles can provide information about concentration, size and refractive index of the particles.

For the scattering signals to provide information about concentration, size and refractive index of the particles, flow cytometry measurements are combined with Mie theory to relate the scattering signal to the particle diameter [8]. Mie theory is used to calculate the angular light scattering by particles. The angular scattering on all detectors is then used to calculate the scattering cross-section of the particle. Using assumptions about input parameters for the refractive index of both the particles as well as the medium surrounding the particles, the scattering signals are related to diameters. Since information on the refractive index of the medium surrounding the particles was lacking, we developed a new measurement set-up for traceable refractive index determination of both liquids and solids [9].

Unique features

Figure 2b shows the features of the metrological flow cytometer, which is not built to measure microparticles such as cells, but for traceable measurements of nanoparticles in fluids. To enable traceable nanoparticle measurement, we provided the metrological flow cytometer with seven technical improvements over commercial flow cytometers. These technical improvements were made from a metrology perspective to measure as accurately as possible, and they would not be feasible on commercial flow cytometers dedicated to measurements of clinical samples. For example, commercial flow cytometers need to measure with a high flow rate, because statistical information about the presence of particles in clinical samples is required.

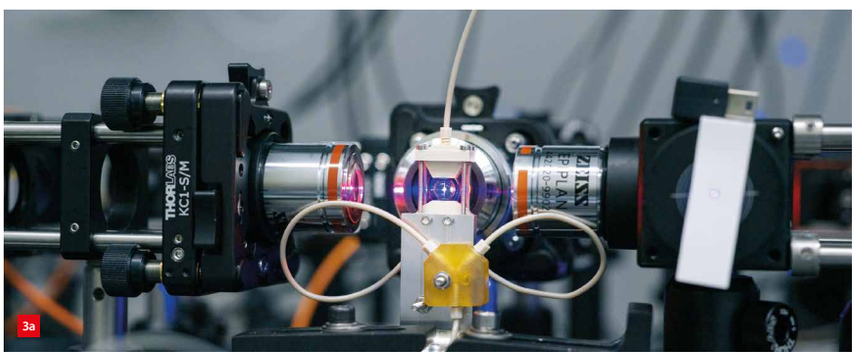




Principle for two types of flow cytometers, differing in beam shape, interrogation volume, flow rate, detectors and fluid cell design; see the text for elaboration.
(a) Commercial flow cytometer.
(b) Metrological flow cytometer.

First, the metrological flow cytometer measures angle-dependent light scattering of single nanoparticles. Commercial flow cytometers cannot distinguish angle-dependent light scattering, since the signal on the photomultiplier tube or photodiode only gives a total scattering intensity. By changing the detectors in the metrological flow cytometer to cameras, we can resolve angle-dependent light scattering. The angle-dependent light scattering is measured in back-, forward-, and side-scattering directions. The spherical shape of the flow cell, shown in Figure 3a, minimises the effect of refraction by the flow cell wall on the measured angle-dependent light scattering.

Second, a Laguerre-Gauss beam illuminates the nanoparticles. Figure 3b shows an example of the (transversal) intensity profile of this specific beam type, which is always donut-shaped, on the forward-scattering camera. The shape of the incoming Laguerre-Gauss beam makes the blocker bar present in commercial flow cytometers unnecessary. A blocker bar is used to block the light coming directly from the laser, such that only the scattered light can be detected. Additionally, the Laguerre-Gauss beam enables us to find unique solutions for the refractive index and size of the particle. When the



Two technical improvements of the metrological flow cytometer.
(a) Spherical flow cell. (Photo: Marcel Cloo)
(b) The Laguerre-Gauss beam, a donut-shaped beam, shown on the camera in forward-scattering direction.

particle is moving through the beam, the particle experiences an angular phase gradient, and gives an interference pattern when not in the exact centre of the Laguerre-Gauss beam [10].

Additionally, the fluidics of the metrological flow cytometer have technical advantages over the fluidics of commercial flow cytometers. So, as a third technical improvement, the metrological flow cytometer has calibrated syringe pumps to control the sheath and sample flow. The syringe pump is calibrated with a relative measurement uncertainty in the flow rate of $2.75 \cdot 10^{-3}$. Fourth, the syringe pump lets the sample flow with a flow rate 125,000-fold lower than in commercial flow cytometers. Fifth, the sample passes through an interrogation volume 500-fold smaller. Sixth, as a result of the low flow and small interrogation volume, single particles can each be measured for half a second, which is a 2,500-fold longer measurement time than in commercial flow cytometers.

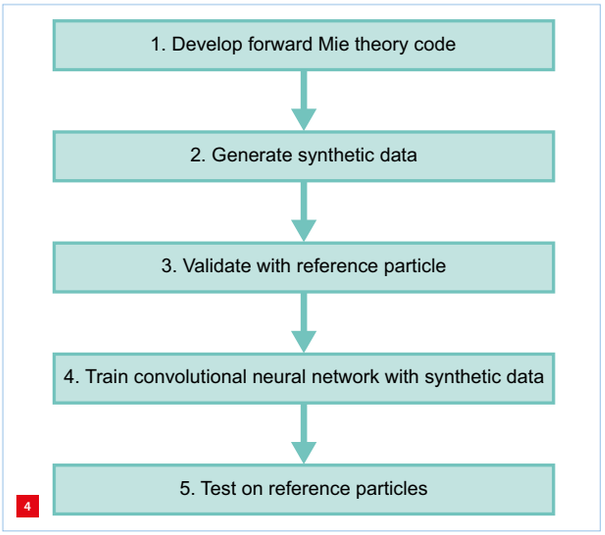
Seventh, a machine learning algorithm, a convolutional neural network, is being developed to relate signals to theory and determine nanoparticle properties. Figure 4 shows the structure used in the development of the convolutional neural network. A forward Mie theory code is developed for generating synthetic data of both solid nanoparticles and core-shell nanoparticles (such as extracellular vesicles) illuminated by a Laguerre-Gauss beam. Using the forward Mie theory code, synthetic data is generated. This data matches the data measured by the metrological flow cytometer as close as possible in terms of background noise and size correlation between the images and the camera.

In the next step, the generated synthetic data is validated using a traceable reference particle of known size and refractive index. When the synthetic data is validated, the data is used to train a convolutional neural network. The convolutional neural network uses the scattering images to determine (i) the radius of the particle, (ii) the refractive index of the particle, (iii) the thickness of the shell, and (iv) the refractive index of the shell in case of core-shell nanoparticles. We will test the convolutional neural network using a set of reference particles, and extend the model to provide an uncertainty analysis of the determination of radius, refractive index and shell thickness.

Using the metrological flow cytometer, we will perform the first traceable concentration, size, and refractive index measurements of nanoparticles.

Applications

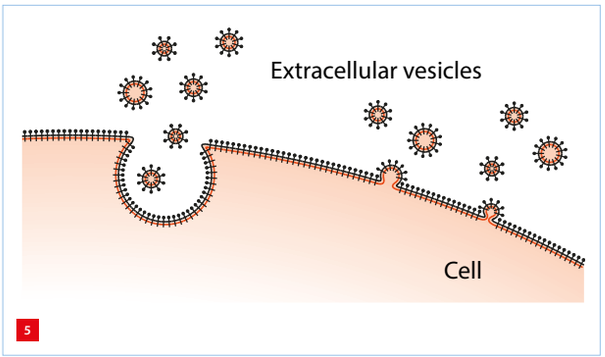
Determination of the physical properties of nanoparticles is in high demand in a broad range of applications, including (i) reference materials [4], (ii) extracellular vesicles [4], (iii) nanoplastics [2], (iv) particulate matter [3], and (v) drug-loading liposomes [5].



Steps performed by the machine learning algorithm to gather size and refractive index information of the nanoparticles.

The first application, the characterisation of reference materials, is highly relevant in metrology, since this is important both for determining the quality of the reference materials and using the reference materials for calibration of apparatus such as commercial flow cytometers. As mentioned above, Mie theory can be used to relate scattering signals to diameter and refractive index.
However, commercial flow cytometers require calibration using reference materials of known size and refractive index before biological samples can be measured. Currently, the properties of biological samples are determined using reference materials of known size and assumed refractive index [4]. By calibrating reference materials on both size and refractive index, nanoparticle properties can be determined without doing assumptions about reference materials.

The second application focusses on the nanoparticles that can be found in the biological samples measured by flow cytometry, namely extracellular vesicles, i.e. nanoparticles released by all cells in the human body, as shown in Figure 5. Extracellular vesicles are present in all body fluids, including blood, urine, saliva and even sweat. Additionally, extracellular



Extracellular vesicles are small nanoparticles released by all cells within the human body.

vesicles have properties that can change with disease, such as differences in concentration, markers on the extracellular vesicle surface or content of the extracellular vesicle. Therefore, extracellular vesicles are an ideal non-invasive biomarker.

However, since extracellular vesicles are so small, mostly below 200 nm, detection of extracellular vesicles has its challenges. Additional challenges arise from the heterogeneity of body fluids, since these contain extracellular vesicles from different origins as well as other particles within the size range of extracellular vesicles [4]. Single detection of extracellular vesicles based on size and refractive index detection can give extra insight into their concentrations, and result in their clinical detection.

The third application is detection of nanoplastics. Worldwide, there is an increasing concern about the effect of plastic pollution on health and environment. Therefore, detection of nanoplastics becomes increasingly important. Nanoplastics can be found in ocean and fresh water [2], soil [11] and even in your blood [12]. Due to their heterogeneity, plastic nanoparticles are difficult to quantify [2]. Therefore, detection of single plastic nanoparticles in liquids would give new insights about the origin of nanoplastics present in water, soil or blood. These new insights could prove crucial in a battle against plastic pollution.

The fourth application in which single-nanoparticle detection would be important is the measurement of particulate matter, particles in air. Particulate matter includes nanoparticles released during mining, combustion, or transportation [3]. While particulate matter is mostly present in air, particulate matter can also be suspended in water [3], or transported to the bloodstream through inhalation [13]. Single-particle detection could give information about the origin of the particulate matter and its spreading, or provide insights for studies about toxicity.

The fifth and final application we will discuss is the use of nanoparticles for drug delivery. Due to their small size, nanoparticles have unique biological, chemical and physical properties, making them ideal drug carriers for precision medicine. Nanoparticles can be engineered to hold certain drugs, and can carry these drugs to the right location in the human body.
There are three classes of nanoparticles for drug delivery: (i) polymeric, (ii) inorganic, and (iii) lipid-based. Using single-particle detection, nanoparticle size and content can be measured. Ideally, the chemical composition of such drug-loaded nanoparticles can be detected. In the future, Raman spectroscopy can be added to the metrological flow cytometer for determining the chemical composition of nanoparticles.

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