Detection of extracellular vesicles: size does matter
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APPENDIX D

Light scattering calculations

Electromagnetic fields, such as light, can be mathematically described by Maxwell’s equations. Mie theory provides an analytical solution of Maxwell’s equations and describes light scattering of spheres and shells of all size parameters. Mie theory is extensively described by Bohren and Huffman [40]. The formulations by Bohren and Huffman are implemented in Matlab by Mätzler [197]. Throughout this thesis, we have used the scripts of Mätzler to calculate the scattering properties of beads, cells, and vesicles.

D.1 Total scattering cross section

The total scattering cross section $C_{\text{sca, tot}}$ is a hypothetical area describing the probability of light with unit incident irradiance being scattered (in all directions) by the particle. Therefore, the scattering cross section is proportional to the quantity of light scattered by a particle. In Chapter 3, we used the scripts of Mätzler to calculate the scattering efficiency $Q$, which is related to $C_{\text{sca, tot}}$ and the particle cross section as follows:

$$Q = \frac{4C_{\text{sca, tot}}}{\pi d^2} \quad (D.1)$$

where $d$ is the particle diameter. We calculated the scattering cross section of a gold sphere ($n_p = 0.40 + 2.54i$) with 200 nm diameter in water ($n_w = 1.33$) using an illumination wavelength of 532 nm to be $1.13 \cdot 10^5 \text{ nm}^2$. The character $i$ is mathematically defined as $i = \sqrt{-1}$ and represents the absorption losses of the material. The scattering cross section for a 200 nm polystyrene sphere ($n_p = 1.60$) is $4.22 \cdot 10^3 \text{ nm}^2$, which is 27 times smaller than the scattering cross section of a 200 nm gold sphere. The scattering cross section for a 200 nm vesicle ($n_p = 1.38$) that contains a 10 nm thick shell ($n_s = 1.46$), e.g. a phospholipid membrane, is 283 nm$^2$, which is 15 times smaller than the scattering cross section of a 200 nm polystyrene sphere and 399 times smaller than that of a 200 nm gold sphere.

D.2 Aperture-weighted scattering cross section

The detection methods used in Chapters 4, 5, and 6 collect scattered light under specific angles. To take into account the optical configuration of these instruments, the differential scattering cross section has to be integrated over the collection
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Figure D.1: Optical configuration of the (A) FACSCalibur flow cytometer and (B) coordinate system and variables used to calculate the scattering intensity of a spherical particle that is illuminated by a laser beam. Symbols are explained throughout the text.

angles of the instrument. In Chapter 5, we followed the approach of Fattaccioli et al. [97] to calculate the forward and side scattered light from beads and vesicles for the BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). However, Fattaccioli et al. did not take into account that the transmission efficiency of a lens decreases with increasing propagation angle relative to the optical axis.

In the next section, we will discuss the approach that we used in Chapters 4 and 6 to calculate the light scattering intensity for particles measured by nanoparticle tracking analysis (NTA; NS500, Nanosight, UK) and flow cytometry.

D.2.1 Optical configuration FACSCalibur

Fig. D.1A shows a schematic of the optical configuration of the FACSCalibur. A linearly polarized 15 mW argon-ion laser emitting at 488 nm is elliptically focused to illuminate cells or vesicles, which are hydrodynamically focused in the flow cell. Forward scattered light (FSC) is measured in line with the laser beam. To prevent the laser directly illuminating the photodiode, the laser beam is blocked by the obscuration bar (OB). Side scattered light (SSC) is collected by an objective with a numerical aperture (NA) of 1.2, which is placed perpendicular to the beam. NA characterizes the range of angles over which the objective collects light and is defined as \( NA = n_m \sin \alpha_{\text{max}} \), where \( n_m \) is the refractive index of the medium and \( \alpha_{\text{max}} \) is the maximum propagation angle. The SSC detector is a photomultiplier tube (PMT), which is not only more sensitive than the photodiode of the FSC detector, but also detects scattered light over a much broader angle. Therefore, we select SSC as the trigger signal and measure SSC from vesicles to derive their particle size distribution (PSD).
D.2. Aperture-weighted scattering cross section

D.2.2 Optical configuration NS500

The optical configuration of the Nanosight NS500 is comparable to the detection of SSC in the FACSCalibur. A linearly polarized 45 mW diode laser emitting at 405 nm is collimated in a flow cell and illuminates vesicles. Light is collected in the SSC direction by an objective with an NA of 0.4.

D.2.3 Model

Fig. D.1B shows a spherical particle with diameter $d$ and refractive index $n_p$ that is illuminated by a plane electromagnetic wave propagating along the $z$ direction and polarized in the $x$ direction:

$$ E_i = E_{0,x} e^{i(kz-\omega t)} \hat{e}_x $$ (D.2)

with $E_{0,x}$ the electric field amplitude, $\omega$ the angular frequency, $t$ the time, $\hat{e}_x$ the orthonormal basis vector in the direction of the positive $x$ axis, $k = 2\pi n_m/\lambda$ the wave number, and $\lambda$ is the wavelength of the incident light in vacuum. The total scattering cross section is given by:

$$ C_{sca} = \int_{0}^{2\pi} \int_{0}^{\pi} \frac{|X|^2}{k^2} \sin \theta d\theta d\phi $$ (D.3)

with $\theta$ the polar angle, $\phi$ the azimuthal angle, and $X$ the vector scattering amplitude. In a flow cytometer, $\theta$ and $\phi$ are limited by the optical aperture of the microscope objective, and since the detected scattering power $P$ is in arbitrary units, a scalar $F$ is introduced to scale the calculations to the data:

$$ P = F \int_{\phi_1}^{\phi_2} \int_{\theta_1}^{\theta_2} \eta \frac{|X|^2}{k^2} \sin \theta d\theta d\phi $$ (D.4)

where $\eta$ is the angle dependent transmission efficiency of the objective. For SSC on the FACSCalibur, $\theta$ is integrated from $\theta_1 = \theta_o - \alpha_{max}$ to $\theta_2 = \theta_o + \alpha_{max}$, with $\theta_o$ the angle between the optical axis of the objective and the propagation direction of the incoming wave $\hat{e}_z$. Since the objective has a circular geometry, $\phi_1$ is expressed in terms of $\theta$ as follows:

$$ \phi_1 = \arcsin \left( \frac{\sin \left( \frac{1}{2} \pi - \alpha_{max} \right)}{\sin \left( \frac{1}{2} \pi - \theta_o + \theta \right)} \right) $$ (D.5)

and $\phi_2 = \pi - \phi_1$. The number of steps over which $\theta$ and $\phi$ are integrated is 50. For a spherical particle, the vector scattering amplitude $X$ is related to the amplitude scattering matrix elements $S_j$ as follows:

$$ X = (S_2 \cos \phi) \hat{e}_{\parallel} + (S_1 \sin \phi) \hat{e}_{\perp} $$ (D.6)

where the basis vector $\hat{e}_{\parallel}$ is parallel and $\hat{e}_{\perp}$ is perpendicular to the scattering plane, which is defined by the scattering direction $\hat{e}_r$ and the propagation direction.
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Figure D.2: Transmission efficiency versus angle of propagation for a high NA objective.

of the wave \( \hat{e}_z \). The parameters \( S_1 \) and \( S_2 \) depend on \( d, n_p, n_m, k, \) and \( \theta \), and are calculated using the Matlab routines of Mätzler. Since \( \hat{e}_{\|s} \) and \( \hat{e}_{\perp s} \) are orthogonal, the term \( |X|^2 \) can be written as

\[
|X|^2 = |S_2|^2 \cos^2 \phi + |S_1|^2 \sin^2 \phi
\]  

(D.7)

Equation (D.7) is used to calculate the scattering power for FSC on the NS500. In the FACSCalibur, however, SSC is filtered by a 90/10 beam splitter placed under the Brewster angle, such that only the scattered component parallel to \( \hat{e}_x \) is measured [97]. Under these conditions, the term \( |X \cdot \hat{e}_x|^2 \) is to be considered and can be written as

\[
|X|^2 = |S_2|^2 \cos^4 \phi \cos^2 \theta + |S_1|^2 \sin^4 \phi + (S_1 S_2^* + S_1^* S_2) \cos^2 \phi \sin^2 \phi \cos \theta
\]  

(D.8)

Since the transmission efficiency \( \eta \) of light propagating through a high NA objective decreases with increasing propagation angle \( \alpha \) with respect to \( \hat{e}_o \) (see Fig. D.1A), a sine function was chosen empirically as a weighting function for \( \eta \):

\[
\eta = \sin \left( \frac{\pi \alpha}{2 \alpha_{max}} + \frac{1}{2} \pi \right)
\]  

(D.9)

Fig. D.2 shows an example of the weighting function for \( \alpha_{max} = 0.35\pi \), which is close to an NA of 1.2. To calculate \( \alpha \), it is expressed in terms of \( \theta \) and \( \phi \) by using the inner product between \( \hat{e}_x \) and \( \hat{e}_o \) and taking \( \phi_o = \frac{1}{2} \pi \), where \( \phi_o \) denotes the angle between \( \hat{e}_o \) and \( \hat{e}_x \), resulting in

\[
\alpha = \arccos[\sin \phi \cos(\theta_o - \theta)]
\]  

(D.10)

To find the optimal values for unknown values of \( F \), NA, \( \theta_o \), and \( \theta_s \), a least square fit was applied on data from beads of known size and refractive index. Since the NS500 is equipped with a low NA objective, no weighting function was applied.