Detection of ultrafine particles in living cells

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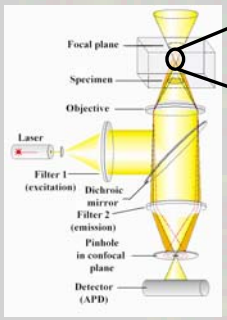
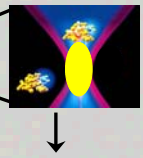
Abstract

If humans are exposed to nanoparticles or nanostructures they can generate general health problems. Our study is directed towards a better understanding of the basic interactions of nanoparticles with cells. The attention should be turned to the general uptake mechanisms. Using different advanced fluorescence methods like Fluorescence Correlation Spectroscopy (FCS) or Photon Counting Histogramming (PCH), allowing the detection of single fluorescent molecules in a defined focus-volume, we could show that nanoparticles of 20nm size occur very fast in the cytoplasm and with a time-delay of about 15 minutes also in the nucleus.

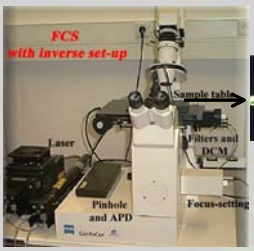


Schematical drawing of the FCS/PCH setup. The laserbeam is sent through a dichroic mirror and an excitation filter (Filter1). After passing the objective (63x, NA 1.2, water) the laser excites particles inside the focus. The fluorophore-labeled particles emit light at a specific wavelength, which passes the dichroic mirror, the emission filter (Filter2) and the pinhole. The emitted photons are detected by a single photon detector (APD). Raw data are used to determine diffusion times and brightness.(picture source: Zeiss GmbH)

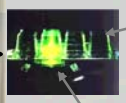


Different particle size and environmental conditions influence diffusion time → **Counting of emitted photons**

Fit of statistical model gives:
- **Number of particles**
- **Brightness of particles**
- **Diffusion time**



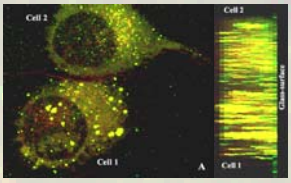
Confocor 1 (Evotec/Zeiss)



Lab Tek chambers

Objective
(Zeiss 63x, NA 1.2, W corr)

Confocal imaging



Confocal view of the uptake of 20nm particles by HeLa cells. Picture A shows a compressed view of 6 nuclear sections (100 nm per layer with a gap of 400 nm). On the right a reconstruction of a side view is shown.

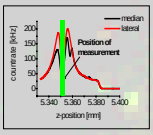
Red ... Cell compartments stained with Nile-Red

Yellow ... Particles or aggregates bound to cellular compounds

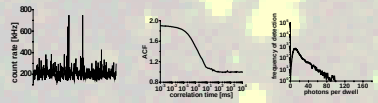
Green ... Isolated particles or aggregates

FCS and PCH

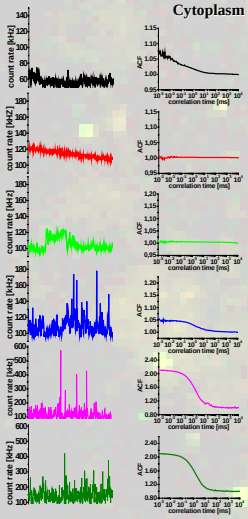
Intensity scan of the cell



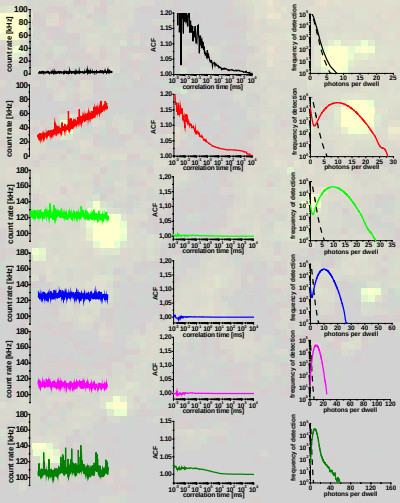
Outside the cell



Cytoplasm



Nucleus



Time-point after addition of particles	τ_1 [ms]	τ_2 [ms]	τ_3 [ms]	intensity [kHz]
1min	0,06 (53%)	1,8 (41%) [Ø≈100nm]	-	57
5 min	no time correlation found			112
10 min	no time correlation found			104
15 min	-	1,4 (76%) [Ø≈40nm]	23,4(23%) [Ø≈1.2µm]	107
35 min	0,001 (17%)	1,4 (78%) [Ø≈40nm]	75 (5%) [Ø≈4µm]	113
60 min	-	1,4 (100%) [Ø≈40nm]	-	145
outside the cell	*0,7 (52%)* [Ø≈40nm]	-	*3 (48%)* [Ø≈160nm]	*631*

Time-point after addition of particles	τ_1 [ms]	τ_2 [ms]	τ_3 [ms]	intensity [kHz]
1 min	no time correlation found			3
5 min	no time correlation found			46
10 min	no time correlation found			123
15 min	no time correlation found			125
35 min	no time correlation found			112
60 min	-	1,05(60%) [Ø≈40nm]	7 (34%) [Ø≈200nm]	109
outside the cell	*0,8 (82%)* [Ø≈40nm]	-	*9,5(15%)* [Ø≈400nm]	*200*

FCS and PCH measurement in the cytoplasm and the nucleus for 20nm polystyrene particles:

Top right: Measuremnet outside the cell. Fluctuations in intensity of up to 400kHz can be detected. The diffusion times range between 0.7 and 3ms depending on aggregation. Emission intensities from 20 to 80 photons per 100 µs are detected. The particle-size ranges from 40 to 160nm in diameter.

Top left: Rapid z-scan through the cell in median and lateral section. The green bar indicates the position of the focus where the measurements are performed.

Bottom left: Measurements in the cytoplasm. At the beginning fluctuations in intensity of 20kHz can be observed, which disappear after 5 minutes. These particles show diffusion times of 1.8 ms and a brightness of 20-35 photons per 100 µs. After 15 minutes fluctuations up to 500kHz appear. Two species of particles can be detected. Their diffusion times range from 1.4 ms for the smaller particles and 23 to 75 ms for larger particles. These species show a brightness of up to 180 photons per 100 µs. The particle-size ranges from 40 nm to 4 µm in diameter (dashed lines indicate control without particles).

Bottom right: Measurements in the nucleus. At the beginning no significant fluorescence intensity can be detected. After 10 minutes a significant increase in intensity can be observed. After 60 minutes fluctuations of 20 – 30 kHz are detected, resulting in diffusion times of 1.05 to 7 ms and a brightness of 50 photons per 100 µs. The particle size ranges from 40 to 400 nm in diameter (dashed lines indicate control without particles).

Introduction

Nanotechnology is widely accepted as a future key technology with a variety of issues for medical treatment and in life sciences. If humans are exposed to nanoparticles, accumulating in the environment from various technological sources as e.g. the exhaust of modern combustion processes, general health problems can be generated.

Our methods allow to follow the time course of the appearance of fluorescent particles in specific compartments in the cell in comparison to the distribution of the particles obtained from confocal scanning microscopy images. The principle for Fluorescence Correlation Spectroscopy (FCS) and Photon Counting Histogramming (PCH) are fluctuations, caused by the passage of fluorescent labeled particles through the focus volume.

Method

HeLa cells (10^5 cells/well) are seeded in LabTek-chambers, using DMEM, supplemented with 4,5mM L-Glutamine, 4500mg/L Glucose, 10% FCS, 10u Penicillin, 10µg Streptomycin and 7,4mg/ml Na-bicarbonate and cultivated over night under standard conditions. The cells are then washed with PBSG (PBS supplemented with 0,02M glucose) and carboxy-coated, green fluorescent particles (yellow-green Fluospheres (510/515), Molecular Probes) of 20nm size were added (0.075w%). The measurement was started immediately after adding the particles.

For the measurement of PCH and FCS the following set-up is used:
Objective: Zeiss 63x; 1.2 W corr
Laser: Ar 480-514nm; 10mW, OD 1.5
Exciting-filter: max: 480-490nm
Dichroic mirror: max: 540-700nm
Emission-filter: max: 520-570nm
Pinhole: 35µm
APD: PerkinElmer SPCM-AQR-13-FC (min 10^6photons/s)
Focus-element: 1.68x0.31µm
Acquisition-time: 30s for PCH and 42s for FCS
Dwell-time for PCH: 100µs
For confocal imaging the following parameters are used:
Pinhole[airy]: 0.465
Layer-size: 100nm
Objective: PL APO 100x, NA 1.4 OIL UV

Results

It could be shown that 20nm carboxy-labeled nanoparticles can be detected in the cytoplasm after a few minutes. The diffusion time of these particles imply that these particles are singular, because of a single diffusion-time and a homogenious distribution of the brightness. After about 30 minutes particles, showing different diffusion-times and brightness distribution, occur. These signals result from aggregation of particles or from particles included in endosomes or vesicles.

Albeit in the nucleus with a time-delay an increase of fluorescence intensity is observed which levels off after aprox. 15 minutes. This signal can be attributed to high concentrations of single diffusing particles. After aprox. 60 minutes additional fluctuations arise which could result from particles attached to macromolecules.

Conclusions and outlook

- **20 nm particles can be detected in the cytoplasm after a few minutes.**
- **Initially isolated particles are observed.**
- **After about 30 minutes larger and brighter particles can be detected, probably included in vesicles.**
- **Accumulation of 20 nm particles can also be observed in the nucleus.**
- **For the uptake of particles, viable cells are necessary.**
- **FCS together with PCH can be used as a fast sreening-method to study interactions of fluorescent labeled particles with cells under physiological conditions.**

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