Image Correlation Spectroscopy with Second Harmonic Generating Nanoparticles in Suspension and in Cells

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Abstract

The absence of photobleaching, blinking, and saturation combined with a high contrast provides unique advantages of higher-harmonic generating nanoparticles over fluorescent probes, allowing for prolonged correlation spectroscopy studies. We apply the coherent intensity fluctuation model to study the mobility of second harmonic generating nanoparticles. A concise protocol is presented for quantifying the diffusion coefficient from a single spectroscopy measurement without the need for separate point-spread-function calibrations. The technique's applicability is illustrated on **nominally 56 nm** LiNbO₃ nanoparticles. We perform label-free raster image correlation spectroscopy imaging in aqueous suspension and spatiotemporal image correlation spectroscopy in A549 human lung carcinoma cells. In good agreement with the expected theoretical result, the measured diffusion coefficient in water at room temperature is $(7.5 \pm 0.3) \mu\text{m}^2/\text{s}$. The diffusion coefficient in the cells is more than 10^3 times lower, and heterogeneous, with an average of $(3.7 \pm 1.5) \times 10^{-3} \mu\text{m}^2/\text{s}$.

Graphical TOC Entry

Fluorescence Correlation Spectroscopy (FCS) has been successfully applied to numerous systems since its first introduction several decades ago.^{1,2} In particular, FCS has been used to study the molecular translational and rotational diffusion properties in solutions and in living cells.^{3–7} In FCS, quantitative information about the dynamics is derived from the temporal fluorescence intensity fluctuations produced by the random movement of fluorophores into and out of the diffraction limited volume of a focused illumination beam. Many variations on this principle exist. Scanning FCS (sFCS) allows measuring slower diffusion rates while reducing photobleaching.^{8–10} FCS in combination with two-photon excitation was developed to measure protein aggregation¹¹ and to probe the mobility of beads in the intracellular transport.¹² In Image Correlation Spectroscopy (ICS), fluctuations in 2D or 3D space are analyzed to quantify the aggregation and the average number of stationary or slowly moving fluorescently labeled structures.^{13,14} Both spatial and temporal intensity information can be combined in Raster ICS (RICS)^{15–17} and in spatiotemporal ICS (STICS).^{18–21} RICS is a variation on sFCS in which the speed of the moving excitation volume in a laser scanning microscope (LSM) is explicitly used to resolve shorter timescale dynamics than covered by STICS. STICS analyzes the spatial and temporal lags in a series of images collected with short acquisition times. This technique not only allows the measurement of the diffusion coefficient but also mapping flow velocities in a wide dynamic range.

However, fluorescence-based methods carry several disadvantages. Long-term studies of both slow and fast processes are impeded, as fluorophores are sensitive to photobleaching, blinking and saturation.^{22–25} In addition, autofluorescence of the environment, which is in particular problematic for imaging in cells and tissues,²⁶ further complicates the use of fluorescent probes.

These drawbacks are not, or at least a lot less, present in Second Harmonic Generation (SHG) imaging. SHG is a nonlinear scattering process, produced in the bulk of non-centrosymmetric crystal structure materials, such as BaTiO₃, LiNbO₃, ZnO and

BiFeO₃.^{22,27,28} The second harmonic signal is extremely stable and strong,²² providing a high contrast in samples that are not SHG active.²⁹ Harmonic nanoparticles (HNPs), *i.e.* higher-harmonic generating nanoparticles (NPs), are therefore ideal for long-term dynamics studies in complex biological specimens.^{22,30–33} Not only can HNPs be employed as optically superior labels, investigating interactions between NPs and human cells is in itself important for *e.g.* nanomaterial safety studies.^{19,27}

The coherent nature of SHG requires a different model for analyzing the observed intensity fluctuations. Geissbuehler *et al.* presented the FCS variant with HNPs circulating through a flow cell, which they termed nonlinear correlation spectroscopy, and derived the corresponding equations for interpreting the data.²³ This model was later extended to a general coherent intensity fluctuation model (cIFM).³⁴ cIFM **comprises a set of formulas describing** the intensity correlation between any two points in space and time assuming free diffusion of the particles, with or without flow superimposed. Using for each technique the appropriate substitution, cIFM can be employed for the coherent counterparts of fluorescence-based correlation spectroscopies FCS, TICS, RICS, and STICS, which were named coherence Correlation Spectroscopy (cCS), cTICS, cRICS, and cSTICS, respectively. The prefix 'c', pronounced as 'coh', stresses the coherent aspect of the scattering process.

Here, we verify cIFM by performing cRICS measurements on SHG active LiNbO₃ and BaTiO₃ HNPs suspended in water and in a water–glycerol mixture. We present a concise protocol for extracting the diffusion coefficient from a single measurement without the need for separate point-spread-function (PSF) calibrations. cSTICS is demonstrated in live adenocarcinomic human alveolar basal epithelial cells (A549 cell line) to illustrate the potential of dynamic SHG correlation spectroscopy measurements in living cells with biocompatible³⁵ HNPs. Quantitative characterization of the mobility of HNPs within living cells and cell organelles allows gaining insight into the complexity of nanoparticle transport.^{32,36}

Typically observed cRICS (56 nm LiNbO₃ nanoparticles) and fluorescence RICS (100 nm

beads) frames for comparison are depicted in Fig. 1 and Fig. S1. Note that the spatially summed SHG intensity over time, plotted in Fig. S2, is very stable, due to the absence of bleaching of the HNPs. The combined movement of the LiNbO_3 particles and the illumination beam results in a collection of stripes in the images, similar to what is observed for the fluorescent beads. There is, however, a difference between both images. While the signal from the beads is often detected in multiple consecutive lines, the HNPs show a more discontinuous pattern, in which a particle can be found in one line before disappearing for some time, and then reappearing some lines later. Evidence for this behavior is provided by the autocorrelation function (ACF) $G(\xi, \psi)$, shown in Fig. 1. The ACF is a function of the spatial lags ξ and ψ in the fast x- and slow y- direction, respectively, which themselves are functions of temporal lags in RICS/cRICS because of the scanning motion of the laser beam. The $\psi = 0$ line corresponds to lag times in the microsecond range, *i.e.* the same order of magnitude as the pixel dwell time. The other lines represent lag times in the millisecond range, *i.e.* the same order of magnitude as the line scan time. The shape of the ACF is affected by both system and sample related parameters. The first class comprises the lateral and axial radius of the PSF, ω_0 and z_0 , respectively, and the Gouy phase shift per unit length κ_q which is present in a focused laser beam.³⁷ The system parameters must be known prior to the analysis of extracting the sample information. The sample related parameters are the concentration $\langle c \rangle$, diffusion coefficient D and directed flow velocity of the particles $\mathbf{v}$. In the experimental ACF shown in Fig. 1, the central line $G(\xi, \psi = 0)$ protrudes above the rest of the curve, with no effect of the selected scan speed, as indicated in Fig. S3. As a control to exclude sample and system related artifacts, we measured the same sample with a different setup (Fig. S4), and different samples (50 nm BaTiO_3 HNPs and 100 nm fluorescent microspheres) with the same setup (Fig. S5–S6). An additional control experiment was performed with immobilized LiNbO_3 HNPs (Fig. S7). The fixed HNPs and the fluorescent beads produce a conventional autocorrelation curve, without the central ridge. Although the effect is less pronounced in the water–glycerol mixture,

all other experiments do show significant $\psi = 0$ peaks, independent of the illumination polarization. Consequently, this behavior must be attributed to the intrinsic movement of the particles. Because the effect is not predicted by the cIFM,³⁴ it cannot be caused by free translational diffusion of the HNPs. The microsecond range pixel dwell time is much shorter than the expected millisecond range diffusion time for the HNPs, which further confirms the lack of free translational movement at the $\psi = 0$ time scale. A possible explanation for the drop in G from $\psi = 0$ to $\psi = \pm 1$ is rotational diffusion. The SHG signal is sensitive to the orientation of the HNP with respect to the polarization of the incident light, see Fig. 2. This angular dependency was well fitted with a $\cos^4$ function corresponding to a dipole response due to the projection of the **optical c-axis of the HNP trigonal crystal structure** on the focal plane. This can be expected for SHG materials belonging to the 3m symmetry class, as has been reported for BiFeO_3 .^{38,39} Even under circularly polarized illumination, the observed intensity depends on the angle between the induced SHG dipole moment and the sample plane.⁴⁰ One could expect to ‘lose’ part of the correlation from the $\psi = 0$ to the $\psi = \pm 1$ line due to changing orientations of the HNPs during a line scan time. A spherical particle with a diameter of 56 nm suspended in water at room temperature has a characteristic rotational diffusion time, *i.e.* the inverse of the rotational diffusion coefficient, of 137 μs . Except for the slowest scan speed, this value is longer than the pixel dwell time but significantly shorter than the characteristic translational diffusion time $\omega_0^2/4D$, which is 14.8 ms. An additional motivation for **the** presence of rotational diffusion is presented in Fig. S8, which shows a cRICS stationary line measurement in which a single line, instead of a raster, was repeatedly scanned. The image shows that while some particles are translating slowly and can be observed in dozens of lines, the resulting traces are still discontinuous, showing an on/off pattern in the y-direction. This behavior may be explained as rotational diffusion of the HNPs combined with the ‘photoselection effect’ shown in Fig. 2. It is unlikely that the missing links in the traces can be attributed to axial translational movement of the HNPs out of the

focal volume. A more plausible explanation is a change of the orientation of the HNPs in which a lower second harmonic signal is produced. The corresponding ACF shows that the track gaps in the vertical time direction result in a strong decrease in G from the $\psi = 0$ to the $\psi = \pm 1$ line, which leads to the presence of the central ridge.

Although the appearance of a central ridge ($\psi = 0$) indicates more complex HNP dynamics at the microsecond time scale than solely translational movement, the absence of noticeable diffusion over several pixel dwell times, even for the slowest scan speeds (Fig. S3), makes the $\psi = 0$ line valuable to obtain an independent calibration of the PSF. Panel (e) of Fig. 1 shows $G(\xi, \psi = 0)$, extracted from the data set from panel (c). The parameters ω_0 and $\langle c \rangle$ were fitted with the cIFM³⁴ with D fixed at $7 \mu\text{m}^2/\text{s}$, but almost identical results were found for $D = 0$. Averaging over 3 measurements at different scan speeds and calculating the standard deviation to determine the confidence intervals yields $\omega_0 = (672 \pm 8) \text{ nm}$ and $\langle c \rangle = (0.47 \pm 0.08) / \mu\text{m}^3$. One of the fit results is plotted in Fig. 1, panel (e). The concentration corresponds to an average of 1.4 particles in the focal volume, but this number may not be fully reliable due to the central ridge, which is not taken into account in the theoretical model. The size of the PSF, however, is in good agreement with a reference measurement with fixed blue beads excited with the same wavelength. The result after analyzing 17 subresolution 100 nm polystyrene beads is $(6.5 \pm 0.3) \times 10^2 \text{ nm}$.

Fixing the height of the focal volume z_0 is necessary to fit the central autocorrelation line. Based on a z-stack of the blue beads, a shape factor z_0/ω_0 of 5.1 was used throughout this work. It should, however, be noted that the value of z_0 does not significantly influence the ACF. E.g. fixing the z_0/ω_0 ratio at 3 yields a fitted ω_0 value within the aforementioned confidence interval. The minor importance of z_0 , combined with the extraction of ω_0 from the central ridge, obviates the need for extra calibration experiments.

The coherent nature of the SHG process affects the shape of the cRICS ACF compared to the fluorescence RICS ACF. The lengthy cRICS ACF,³⁴ which was used to fit the data, can be written in a compact form as

$$G(\xi, \psi, \omega_0, z_0, \kappa_q, \langle c \rangle, D) = \frac{A3 + A4 + A5 + A7 + B6 + B7 + B9 + C5 + C7}{G_N}, \quad (1)$$

in which all terms in the numerator are functions of the system parameters ω_0 , z_0 and κ_q and the sample parameters $\langle c \rangle$ and D . G_N is a normalization factor. The sum $(A7 + B9 + C7)/G_N$ is equal to the fluorescence expression. The contribution of the other terms to G increases with increasing particle concentration. Consequently, when using the fluorescence expression to fit the $\psi = 0$ line, a significantly higher value of $(865 \pm 4) \text{ nm}$ is found for ω_0 . This result indicates the importance of using the cIFM for SHG active specimens.

The lateral size of the PSF derived from the central line of the ACF can be employed to calculate the diffusion coefficient from the $\psi \neq 0$ lines. Since the rotational diffusion time is short with respect to the line scan time, the $\psi \neq 0$ lines are not influenced by rotational diffusion. Fixing all parameters, except $\langle c \rangle$ and D , yields a diffusion coefficient of $(7.5 \pm 0.3) \mu\text{m}^2/\text{s}$, averaged over four measurements. This result corresponds to the value predicted by the Stokes–Einstein equation, which is $(8 \pm 2) \mu\text{m}^2/\text{s}$ for particles with a diameter between 45.5 nm and 65.5 nm suspended in water at room temperature (viscosity $1.002 \text{ mPa}\cdot\text{s}$ ⁴¹). The quality of the fit is illustrated by the fit residuals of Fig. 3. **A schematic summary of the data analysis protocol is presented in Fig. S9.**

A diffusion coefficient of $(12 \pm 4) \mu\text{m}^2/\text{s}$ is found upon analysis of the same data sets with the fluorescence expression and by fixing ω_0 at 672 nm . In addition to substantially overestimating D , the non-random structure of the fit residuals, shown in the central panel of Fig. 3, clearly demonstrates the undesirable quality of this fit result.

The fluorescence model may, however, be used to examine SHG data if ω_0 is first calculated from the $\psi = 0$ line by means of the fluorescence expression. Even though this method yields a value of $\omega_0 = (865 \pm 4) \text{ nm}$, which does not correspond to the real PSF

size, the fitted diffusion coefficient, $(7.6 \pm 0.6) \mu\text{m}^2/\text{s}$, closely matches the result obtained from the cIFM. Furthermore, the quality of the fit is, as illustrated in the right panel of Fig. 3, comparable to the SHG analysis.

One of the main advantages of using SHG active materials for mobility studies is the absence of photobleaching, as illustrated by Fig. S2. Consequently, cSTICS enables long-term mapping of the diffusion coefficient in cells, tissues, and living organisms. To check the HNP uptake by the cells, we imaged cells that had been exposed to the LiNbO_3 HNPs for 24 *h*, see Fig. S11. The z-stack in the last panel of Fig. S11 shows that the HNPs are really present inside the cell. A cSTICS example is presented in Fig. 4 and Table 1. We analyzed 3 regions inside the cell, which revealed a non-homogeneous diffusion coefficient. The ACF for region 1 is plotted in Fig. S12. No central ridge is observed, which can be attributed to clustering of the HNPs, thereby reducing the SHG intensity dependence on the orientation of the excitation polarization plane. The corresponding diffusion coefficient is $3.8 \times 10^{-3} \mu\text{m}^2/\text{s}$, which is several orders of magnitude lower than what is observed in aqueous suspension. Similar values are found for the other regions: the diffusion coefficient and standard deviation averaged over 18 regions spread over 4 cells yields $D = (3.7 \pm 1.5) \times 10^{-3} \mu\text{m}^2/\text{s}$. Fitting again the data with the fluorescence expression and a PSF width of $\omega_0 = 875 \text{ nm}$ yields an almost identical result with a D value of $(3.8 \pm 1.8) \times 10^{-3} \mu\text{m}^2/\text{s}$.

Table 1: Diffusion coefficient heterogeneity for the regions of Fig. 4.

Region	Diffusion coefficient [$\times 10^{-3} \mu\text{m}^2/\text{s}$]
1	3.8
2	6.7
3	2.6

The small diffusion coefficient in comparison to the cRICS result cannot only be explained by clustering of the particles during the 24 hours exposure time, since the cluster diameter would theoretically need to be about 1000 times larger than an individual particle.

Instead, the movement of most HNPs must be confined to cellular organelles and the measured diffusion coefficients reflect the mobility of these organelles. The diffusion coefficients we measured are in close proximity to the values found with conventional STICS.²¹

We measured the colocalization of the HNPs with fluorescently labeled lysosomes ($N = 7$) and endosomes ($N = 5$), see Fig. S13. The Manders' coefficients were found to be (0.7 ± 0.2) and (0.6 ± 0.2) , respectively. These results suggest uptake and clustering of the particles inside both cellular organelles. No directed flow of the HNPs could be found within the accuracy of the cSTICS experiments, indicating the absence of active transport of the organelles. As a second test, we checked specifically for any transport of the organelles via the actin microfilaments and the microtubules by adding latrunculin and nocodazole, respectively, to the cells. Each component changes the polymerization state of the assembly that is affected, thereby severely hindering any transport along these structures.^{42,43} Although analysis of 11 regions in 4 cells treated with latrunculin – $D = (1.0 \pm 1.3) \mu\text{m}^2/\text{s}$ – and 15 regions in 4 cells treated with nocodazole – $D = (2.1 \pm 1.6) \mu\text{m}^2/\text{s}$ – both yield lower diffusion coefficients than untreated cells, the spread in D between different regions is too large to draw conclusions on the mechanisms involved in the HNP transport.

In summary, we have delineated the cIFM conditions needed to analyze autocorrelation spectroscopy measurements with second harmonic generating light scattering HNPs. The central line from the cRICS autocorrelation curve surprisingly protrudes above the rest of the surface. This central ridge was not predicted by the theory. Analyzing a cRICS data set therefore requires two steps: the central line is fitted separately and is used to extract the beam waist while the diffusion coefficient is obtained from the other lines. No additional PSF calibrations are needed for this type of experiment. The measured diffusion coefficient for 56 nm LiNbO₃ HNPs suspended in water at room temperature corresponds to the expected value based on the Stokes–Einstein equation. A cSTICS measurement in A549 cells revealed a much lower and heterogeneous mobility, caused

by the uptake of the HNPs in cell organelles, such as endosomes and lysosomes. Instead of using the complex cIFM to analyze the ACF, we have shown that for our experiments the fluorescence expression yields the same diffusion coefficient, provided that the beam waist is also measured using the fluorescence model. Further exploration is required to check whether this bypass can be generalized to other systems or samples. On the one hand, typical challenges with fluorescence RICS and STICS, such as photobleaching and blinking, are absent in higher-harmonic generating materials. On the other hand, the signal-to-noise ratio may be a concern in small nanoparticles. Assuming that a very small size preserves the SHG generating capability, the SHG signal strength declines with a decreasing size of the particles,⁴⁴ scaling as the square of the HNP volume. A HNP with half the radius used in the current manuscript will thus have its SHG strength reduced by a factor of 64. Consequently, the SHG signal may drop below the noise level. Increasing the illumination power is only feasible to some extent since intensities too high will negatively affect the sample. Absorption of the illumination light may lead to local heating of the sample, resulting in convection of the particle suspensions and a non-Brownian motion. Furthermore, high levels of radiation will damage a biological cell. However, it may not be necessary to increase the illumination power. Kim *et al.* were able to detect the second harmonic signal generated by a single 22 nm BaTiO₃ HNP, indicating their potential as biomarkers.⁴⁴ In a more elaborate approach that might be applicable to some cases, the SHG intensity may be enhanced by a factor of more than 500 by using plasmonic core-shell nanocavities, *i.e.* SHG nanocrystals surrounded by a metal shell.⁴⁵

Our findings to employ these HNPs for autocorrelation spectroscopy studies will aid specific applications for long-term, deep tissue and organoid imaging, tissue engineering, chronic wound-healing, and environmental HNPs exposure studies.

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Supporting Information Available

- Slenders_et_al_SI.pdf: this document contains a detailed 'materials and methods' section, additional figures, the autocorrelation sorting protocol and a table with the instrumental settings used in the cRICS measurements.

References

- (1) Magde, D.; Webb, W. W.; Elson, E. Thermodynamic Fluctuations in a Reacting System - Measurement by Fluorescence Correlation Spectroscopy. *Physical Review Letters* **1972**, 29, 705–708.
- (2) Elson, E. L.; Magde, D. Fluorescence Correlation Spectroscopy. I. Conceptual Basis and Theory. *Biopolymers* **1974**, 13, 1–27.

- (3) Rigler, R.; Grasselli, P.; Ehrenberg, M. Fluorescence Correlation Spectroscopy and Application to the Study of Brownian-Motion of Biopolymers. *Phys. Scr.* **1979**, *19*, 486–490.
- (4) Palmer, A. G.; Thompson, N. L. Molecular Aggregation Characterized by High-Order Autocorrelation in Fluorescence Correlation Spectroscopy. *Biophys. J.* **1987**, *52*, 257–270.
- (5) Kask, P.; Piksarv, P.; Mets, U.; Pooga, M.; Lippmaa, E. Fluorescence Correlation Spectroscopy in the Nanosecond Time Range - Rotational Diffusion of Bovine Carbonic Anhydrase-B. *Eur. Biophys. J. Biophys. Lett.* **1987**, *14*, 257–261.
- (6) Schwille, P.; Korlach, J.; Webb, W. W. Fluorescence Correlation Spectroscopy with Single-Molecule Sensitivity on Cell and Model Membranes. *Cytometry* **1999**, *36*, 176–182.
- (7) Brock, R.; Hink, M. A.; Jovin, T. M. Fluorescence Correlation Microscopy of Cells in the Presence of Autofluorescence. *Biophys. J.* **1998**, *75*, 2547–2557.
- (8) Weissman, M.; Schindler, H.; Feher, G. Determination of Molecular-Weights by Fluctuation Spectroscopy - Application to DNA. *P. Natl. Acad. Sci. USA* **1976**, *73*, 2776–2780.
- (9) Petersen, N. O. Scanning Fluorescence Correlation Spectroscopy 1. Theory and Simulation of Aggregation Measurements. *Biophys. J.* **1986**, *49*, 809–815.
- (10) Ruan, Q. Q.; Cheng, M. A.; Levi, M.; Gratton, E.; Mantulin, W. W. Spatial-Temporal Studies of Membrane Dynamics: Scanning Fluorescence Correlation Spectroscopy (Sfcs). *Biophys. J.* **2004**, *87*, 1260–1267.
- (11) Berland, K. M.; So, P. T. C.; Chen, Y.; Mantulin, W. W.; Gratton, E. Scanning Two-Photon Fluctuation Correlation Spectroscopy: Particle Counting Measurements for Detection of Molecular Aggregation. *Biophys. J.* **1996**, *71*, 410–420.

- (12) Berland, K. M.; So, P. T. C.; Gratton, E. 2-Photon Fluorescence Correlation Spectroscopy - Method and Application to the Intracellular Environment. *Biophys. J.* **1995**, *68*, 694–701.
- (13) Petersen, N. O.; Hoddellius, P. L.; Wiseman, P. W.; Seger, O.; Magnusson, K. E. Quantitation of Membrane-Receptor Distributions by Image Correlation Spectroscopy - Concept and Application. *Biophys. J.* **1993**, *65*, 1135–1146.
- (14) Kolin, D. L.; Wiseman, P. W. Advances in Image Correlation Spectroscopy: Measuring Number Densities, Aggregation States, and Dynamics of Fluorescently Labeled Macromolecules in Cells. *Cell Biochem. Biophys.* **2007**, *49*, 141–164.
- (15) Digman, M. A.; Sengupta, P.; Wiseman, P. W.; Brown, C. M.; Horwitz, A. R.; Gratton, E. Fluctuation Correlation Spectroscopy with a Laser-Scanning Microscope: Exploiting the Hidden Time Structure. *Biophys. J.* **2005**, *88*, L33–L36.
- (16) Rossow, M. J.; Sasaki, J. M.; Digman, M. A.; Gratton, E. Raster Image Correlation Spectroscopy in Live Cells. *Nat. Protoc.* **2010**, *5*, 1761–1774.
- (17) Digman, M. A.; Gratton, E. Analysis of Diffusion and Binding in Cells Using the RICS Approach. *Microsc. Res. Tech.* **2009**, *72*, 323–332.
- (18) Hebert, B.; Costantino, S.; Wiseman, P. W. Spatiotemporal Image Correlation Spectroscopy (STICS) Theory, Verification, and Application to Protein Velocity Mapping in Living Cho Cells. *Biophys. J.* **2005**, *88*, 3601–3614.
- (19) Schumann, C.; Schubbe, S.; Cavalius, C.; Kraegeloh, A. A Correlative Approach at Characterizing Nanoparticle Mobility and Interactions after Cellular Uptake. *J. Biophotonics* **2012**, *5*, 117–127.
- (20) Wiseman, P. W. Image Correlation Spectroscopy: Mapping Correlations in Space, Time, and Reciprocal Space. *Methods Enzymol.* **2013**, *518*, 245–267.

- (21) Deville, S.; Penjweini, R.; Smisdom, N.; Notelaers, K.; Nelissen, I.; Hooyberghs, J.; Ameloot, M. Intracellular Dynamics and Fate of Polystyrene Nanoparticles in A549 Lung Epithelial Cells Monitored by Image (Cross-) Correlation Spectroscopy and Single Particle Tracking. *Biochim. Biophys. Acta, Mol. Cell Res.* **2015**, *1853*, 2411–2419.
- (22) Pantazis, P.; Maloney, J.; Wu, D.; Fraser, S. E. Second Harmonic Generating (SHG) Nanoprobes for in Vivo Imaging. *P. Natl. Acad. Sci. USA* **2010**, *107*, 14535–14540.
- (23) Geissbuehler, M.; Bonacina, L.; Shcheslavskiy, V.; Bocchio, N. L.; Geissbuehler, S.; Leutenegger, M.; Marki, I.; Wolf, J. P.; Lasser, T. Nonlinear Correlation Spectroscopy (NLCS). *Nano Lett.* **2012**, *12*, 1668–1672.
- (24) Dempsey, W. P.; Fraser, S. E.; Pantazis, P. SHG Nanoprobes: Advancing Harmonic Imaging in Biology. *BioEssays* **2012**, *34*, 351–360.
- (25) Broillet, S.; Sato, A.; Geissbuehler, S.; Pache, C.; Bouwens, A.; Lasser, T.; Leutenegger, M. Optical Coherence Correlation Spectroscopy (OCCS). *Opt. Express* **2014**, *22*, 782–802.
- (26) Roederer, M.; Murphy, R. F. Cell-by-Cell Autofluorescence Correction for Low Signal-to-Noise Systems - Application to Epidermal Growth-Factor Endocytosis by 3t3 Fibroblasts. *Cytometry* **1986**, *7*, 558–565.
- (27) Staedler, D.; Magouroux, T.; Hadji, R.; Joulaud, C.; Extermann, J.; Schwungi, S.; Passemard, S.; Kasparian, C.; Clarke, G.; Gerrmann, M. et al. Harmonic Nanocrystals for Biolabeling: A Survey of Optical Properties and Biocompatibility. *ACS Nano* **2012**, *6*, 2542–2549.
- (28) Clarke, G.; Rogov, A.; McCarthy, S.; Bonacina, L.; Gun'ko, Y.; Galez, C.; Le Dantec, R.; Volkov, Y.; Mugnier, Y.; Prina-Mello, A. Preparation from a Revisited Wet Chemical Route of Phase-Pure, Monocrystalline and SHG-Efficient BiFeO₃ Nanoparticles for Harmonic Bio-Imaging. *Sci. Rep.* **2018**, *8*, 10473.

- (29) Culic-Viskota, J.; Dempsey, W. P.; Fraser, S. E.; Pantazis, P. Surface Functionalization of Barium Titanate SHG Nanoprobes for in Vivo Imaging in Zebrafish. *Nat. Protoc.* **2012**, *7*, 1618–1633.
- (30) Grange, R.; Lanvin, T.; Hsieh, C. L.; Pu, Y.; Psaltis, D. Imaging with Second-Harmonic Radiation Probes in Living Tissue. *Biomedical Optics Express* **2011**, *2*, 2532–2539.
- (31) Bonacina, L. Nonlinear Nanomedicine: Harmonic Nanoparticles toward Targeted Diagnosis and Therapy. *Mol. Pharmaceutics* **2013**, *10*, 783–792.
- (32) Macias-Romero, C.; Didier, M. E. P.; Zubkovs, V.; Delannoy, L.; Dutto, F.; Radenovic, A.; Roke, S. Probing Rotational and Translational Diffusion of Nanodoublets in Living Cells on Microsecond Time Scales. *Nano Lett.* **2014**, *14*, 2552–2557.
- (33) Dubreil, L.; Leroux, I.; Ledevin, M.; Schleider, C.; Lagalice, L.; Lovo, C.; Fleurisson, R.; Passemard, S.; Kilin, V.; Gerber-Lemaire, S. et al. Multi-Harmonic Imaging in the Second Near-Infrared Window of Nanoparticle-Labeled Stem Cells as a Monitoring Tool in Tissue Depth. *ACS Nano* **2017**, *11*, 6672–6681.
- (34) Slenders, E.; vandeVen, M.; Hooyberghs, J.; Ameloot, M. Coherent Intensity Fluctuation Model for Autocorrelation Imaging Spectroscopy with Higher Harmonic Generating Point Scatterers—a Comprehensive Theoretical Study. *Phys. Chem. Chem. Phys.* **2015**, *17*, 18937–18943.
- (35) Li, J. H.; Qiu, J. C.; Guo, W. B.; Wang, S.; Ma, B. J.; Mou, X. N.; Tanes, M.; Jiang, H. D.; Liu, H. Cellular Internalization of LiNbO₃ Nanocrystals for Second Harmonic Imaging and the Effects on Stem Cell Differentiation. *Nanoscale* **2016**, *8*, 7416–7422.
- (36) Panariti, A.; Misericocchi, G.; Rivolta, I. The Effect of Nanoparticle Uptake on Cellular Behavior: Disrupting or Enabling Functions? *Nanotechnol., Sci. Appl.* **2012**, *5*, 87–100.

- (37) Feng, S. M.; Winful, H. G. Physical Origin of the Gouy Phase Shift. *Opt. Lett.* **2001**, *26*, 485–487.
- (38) Schmidt, C.; Riporto, J.; Uldry, A.; Rogov, A.; Mugnier, Y.; Le Dantec, R.; Wolf, J. P.; Bonacina, L. Multi-Order Investigation of the Nonlinear Susceptibility Tensors of Individual Nanoparticles. *Sci. Rep.* **2016**, *6*, 9.
- (39) Riporto, J.; Demierre, A.; Kilin, V.; Balciunas, T.; Schmidt, C.; Campargue, G.; Urbain, M.; Baltuska, A.; Le Dantec, R.; Wolf, J. P. et al. Bismuth Ferrite Dielectric Nanoparticles Excited at Telecom Wavelengths as Multicolor Sources by Second, Third, and Fourth Harmonic Generation. *Nanoscale* **2018**, *10*, 8146–8152.
- (40) Hsieh, C. L.; Pu, Y.; Grange, R.; Psaltis, D. Second Harmonic Generation from Nanocrystals under Linearly and Circularly Polarized Excitations. *Opt. Express* **2010**, *18*, 11917–11932.
- (41) Lide, D. R. *CRC Handbook of Chemistry and Physics*; CRC Press, 2005.
- (42) Coue, M.; Brenner, S. L.; Spector, I.; Korn, E. D. Inhibition of Actin Polymerization by Latrunculin-A. *FEBS Lett.* **1987**, *213*, 316–318.
- (43) Kulkarni, R. P.; Wu, D. D.; Davis, M. E.; Fraser, S. E. Quantitating Intracellular Transport of Polyplexes by Spatio-Temporal Image Correlation Spectroscopy. *P. Natl. Acad. Sci. USA* **2005**, *102*, 7523–7528.
- (44) Kim, E.; Steinbruuk, A.; Buscaglia, M. T.; Buscaglia, V.; Pertsch, T.; Grange, R. Second-Harmonic Generation of Single Batio₃ Nanoparticles Down to 22 Nm Diameter. *ACS Nano* **2013**, *7*, 5343–5349.
- (45) Pu, Y.; Grange, R.; Hsieh, C. L.; Psaltis, D. Nonlinear Optical Properties of Core-Shell Nanocavities for Enhanced Second-Harmonic Generation. *Physical Review Letters* **2010**, *104*, 4.

Figure 1: (a) cRICS frame with LiNbO_3 HNPs suspended in water at room temperature. Experimental settings: pixel size $\delta r = 166 \text{ nm}$, 256×256 pixels, pixel dwell time $8.19 \mu\text{s}$, $20\times/\text{NA } 0.8$ objective, Mai Tai at 810 nm , 83 mW on the stage, circularly polarized light, sample at room temperature. SHG detection in the BP400–410 nm channel. (b) RICS frame with 100 nm blue fluorescent carboxylated microspheres. Laser power 70 mW on the stage. Fluorescence detection in the BP450–650 nm channel. All other experimental settings identical to panel (a). Scale bars $10 \mu\text{m}$. (c) cRICS autocorrelation curve. **For representation purposes, the $G(0, 0)$ noise peak was removed from the plot by setting this value to $G(1, 0)$.** From the time series of 800 frames, the 200 best autocorrelation curves were averaged. (d) $G(\xi = 1, \psi)$ cross-section. The cross-section $G(\xi = 0, \psi)$ cannot be used to illustrate the central ridge due to the noise peak at $G(\xi = 0, \psi = 0)$. (e) $G(\xi, \psi = 0)$ cross-section, with the three central points omitted, and the cIFM fit. The fitted parameter values are the PSF width $\omega_0 = 676 \text{ nm}$ and the average particle concentration $\langle c \rangle = 0.41 / \mu\text{m}^3$.

Figure 2: (a) Illustration of the SHG intensity as a function of the orientation of the illumination polarization plane for fixed LiNbO₃ HNPs. The second harmonic signal generated by the HNP is brightest when the polarization plane of the incident light is aligned with the dipole moment induced in the HNP. Almost no SHG is detected when the illumination polarization plane is rotated over 90°. Scale bar 1 μm . (b) Illustration of the LiNbO₃ SHG intensity as a function of the orientation of the illumination polarization plane for three spots, indicated with the different colors. The polarization plane was rotated in steps of 20°. Angles are defined as counterclockwise rotations with the zero angle being along the horizontal. The continuous lines show the $\cos^4$ fits.

Figure 3: LiNbO₃ cRICS fit residuals from the ACFs from Fig. 1 using (a) the cIFM with the PSF width ω_0 fixed at 672 nm and (b–c) using the fluorescence model with (b) $\omega_0 = 672$ nm and with (c) $\omega_0 = 865$ nm. The corresponding diffusion coefficients averaged over 4 measurements are $(7.5 \pm 0.3) \mu\text{m}^2/\text{s}$, $(12 \pm 4) \mu\text{m}^2/\text{s}$ and $(7.6 \pm 0.6) \mu\text{m}^2/\text{s}$. The reduced χ^2 values are 1.3, 3.3 and 1.5, respectively.

Figure 4: cSTICS measurement of LiNbO_3 HNPs (green) in an A549 cell (transmission image). See Table 1 for the measured diffusion coefficients of the different regions. Scale bar $10\ \mu\text{m}$.