# Advancing fluorescence microscopy and spectroscopy for the benefit of cell science and drug delivery

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**Abstract.** Here we report on two recent advances we made applying advanced fluorescence microscopy and spectroscopy to further the study of cell biology and drug delivery. At first, we detail a new instrumental set-up combining atomic force microscopy in liquid and super-resolution fluorescence microscopy in novel configuration such that long-term simultaneous and co-localized observation with both techniques becomes feasible. We believe this will contribute to the study of fast membrane activated cell-signalling processes in the years to come. Furthermore, we report on a novel application of fluorescence cross-correlation spectroscopy (FCCS) for the characterization of lipid-nucleic acid complexes. We could determine the number of nucleic acid particles incorporated in each liposome, a parameter not readily accessible on ensemble basis with other methods. This parameter is crucial for co-delivery applications, and we believe FCCS can play an important role in the future development of new drug delivery systems.

## 1 Introduction

In this work we present the first demonstration of a hardware set-up capable of performing simultaneously super-resolution fluorescence microscopy and atomic force microscopy (AFM). We combine AFM with super-resolved structured illumination microscopy (SR-SIM), a super-resolution technique that allows to surpass the diffraction barrier by a factor of two in every spatial direction. In this set-up it is possible to acquire AFM data under constant SIM illumination without inducing distortions on the AFM cantilever. AFM features high-resolution imaging and quantitative nanomechanical analysis of biological samples, requiring minimum to no specimen modification, further enabling measurements at near physiological/native conditions. Due to its surface characterization nature, AFM is further combined with other microscopy modalities, thus unlocking a truly correlative potential for simultaneous acquisition of physical, chemical and biological information in complex systems.

We also present the successful application of dual-color fluorescence cross-correlation spectroscopy (FCCS) as a direct method to characterize and optimize the assembly of nonviral cationic liposome (CL)-DNA complexes based on a CL formulation consisting of the cationic lipid DOTAP and zwitterionic lipid DOPC [2]. We demonstrate that FCCS is able to quantitatively determine the extent of the association between DNA and the liposomes and assess its loading capacity.

## 2 Combining Super-Resolution Optical Microscopy and Atomic Force Microscopy

The schematic representation of the experimental set-up of the combined SR-SIM-AFM combined platform is depicted in Fig. 1. It is based on an atomic force microscope (JPK NanoWizard 3, Bruker Nano GmbH,

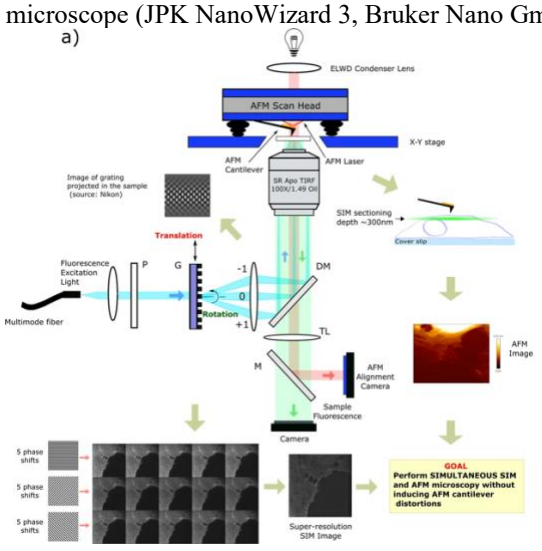


**Fig. 1.** Experimental set-up for the SR-SIM and AFM combined platform. Adapted from [1].

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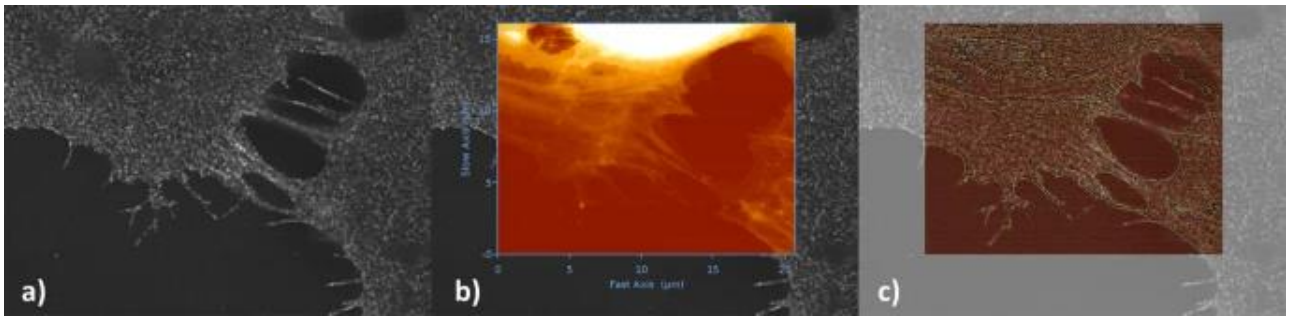


**Fig. 2.** SR-SIM images of the human bone osteosarcoma epithelial cells (a) superimposed with the height/topography channel of the AFM data (b). The correlated SR-SIM/AFM channels on cells in a semi-transparent mode (XY-scales of the AFM image in (b) (c), include a mean-curvature enhanced AFM-topographic information [1].

Berlin, Germany) and a structured illumination microscope (N-SIM E, Nikon Instruments Europe B.V., Amsterdam, The Netherlands). The AFM is mounted on an inverted microscope (Eclipse Ti2-E, Nikon Instruments Europe B.V., Amsterdam, The Netherlands) and a standard monochrome CCD camera (ProgRes MFCool, Jenoptik, Jena, Germany) is coupled for AFM laser spot cantilever alignment.

System performance is demonstrated by conducting simultaneous operation of SR-SIM/AFM using CRISPR/Cas9 genome-edited human bone osteosarcoma epithelial cells, with plasma membrane transporter 1 (MCT1) tagged with an enhanced green fluorescent protein (EGFP) at the N-terminal (Fig. 2).

## 3 Fluorescence cross-correlation spectroscopy for lipoplex characterization

Among nonviral vehicles for delivery applications, cationic liposome-DNA (CLDNA or lipoplexes) nanoparticles are among the most promising since they present low immunogenicity, potential for large-scale production and high flexibility of functionalization. FCSS is an optical technique that by labelling DNA and liposomes using green and red dies, respectively, is able to provide information the time traces generated by the two fluorescently labelled species simultaneously, enabling the study of specific bimolecular binding.

In the experiments, DNA was incubated for at least 30 minutes with green fluorescent dye YOYO-1 prior to use. For lipoplexes, Texas red DHPE was used as labelling agent. FCCS measurements were conducted using a Zeiss LSM 780-NLO confocal microscope (Carl Zeiss AG, Jena, Ger- many) with a Zeiss C-Apochromat 40×/1.2 W objective. YOYO-1 fluorescence was excited using the 488 nm line of the Argon (Ar) laser and Texas Red was excited using the 561 nm diode-pumped solid state (DPSS) laser.

## 4 Conclusions

In this work we present the first demonstration of a hardware set-up capable of performing simultaneously super-resolution fluorescence microscopy and AFM. We combine AFM with SR-SIM, a super-resolution technique that allows to surpass the diffraction barrier by a factor of two in every spatial direction. In this set-up it is possible to acquire AFM data under constant SIM illumination without distorting the cantilever operation. We believe that this scheme combination of microscopy techniques presents one of the most promising schemes enabling simultaneous co-localized imaging, allowing the recording of nanomechanical data and cellular dynamics at the same time.

Moreover, we report for the first time on the potential of fluorescence cross-correlation spectroscopy for the characterization of previously unavailable properties of cationic lipid-DNA complexes. This methodology promises to become a powerful guide to the development of similar formulations and accelerate research and innovation in mRNA vaccine and cancer vaccine development. As an example, in a recent development as a follow-up of this research, we report on the discovery of a new stability criterion for the preparation of stable hybrid lipid-polymer-nucleic acid nanoparticles [3].

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