Imaging quantum dots on the surface of cells

Introduction
Microscopic imaging of cells can generate many different kinds of information about a sample, as determined by the mode of contrast used. By combining microscopy methods, multiple channels can be acquired, generating information on multiple sample properties. The combination of simultaneous fluorescence and atomic force microscopy (AFM) has been used to characterise cellular structures [1, 2], link structure (detected with AFM) to function (by localising specific molecules) [3] and to simultaneously obtain information about the chemical and structural nature of a sample [4]. In each case, the fluorescence was obtained using chemical fluorophores. However, fluorescence labelling can also be achieved using quantum dots. The use of quantum dots for labelling biological structures has many potential advantages, including the fact that the structure of the quantum dot may also be detected using AFM. In such a way it is possible to look at whether the label is present inside or outside a cell, or whether labelled proteins are clustered. The JPK Nanowizard® is optimised for such experiments, designed as it is to render high quality images of a variety of samples, from very small structures such as quantum dots, up to whole cells. Additionally, the Nanowizard® is designed to fully integrate into an inverted light microscope, without reducing the functionality of either the light microscope or the atomic force microscope.

Quantum dots
Quantum dots are a special type of semi-conductor, unique due to their size (approx 2-10 nm). At such small sizes the semi-conductor material of the quantum dot behaves differently to other forms of semi-conductors. In a bulk semi-conductor material (much larger than a quantum dot) the electrons will occupy multiple energy levels. These energy levels are, in general, so close that they are considered continuous. However, there are some energy levels that the electrons cannot occupy, collectively known as the band gap. Most electrons occupy energy levels below this band gap in an area known as the valence band, indeed most energy levels in the valence band are occupied. If, however, an external stimulus is applied, an electron may move from the valence band to the conduction band i.e. those energy levels above the band gap. The electron in the conduction band and the hole it has left in the valence band are collectively known as an exciton. Energy is then released in the form of electromagnetic radiation as the electron falls back across the band gap to the valence band. The wavelength of this radiation corresponds to the energy the electron loses.

![Semi-conductor energy levels](image)

However, the small size of quantum dots leads to what is known as quantum confinement. This means that the energy levels that the electrons can inhabit become discrete, with a finite separation between them. This then affects the absorption and emission of energy of the semi-conductor material. When excited, the size of the quantum dot results in the emission of light at a single wavelength that is dependent on dot size. By adding or subtracting a single atom this emission wavelength can be changed, with an increase in size leading to a shift toward longer wavelengths, i.e. the red end of the visible spectrum.

The optical properties of quantum dots will change with proximity of quantum dots to each other. The combined use of fluorescence and atomic force imaging makes such studies possible. The JPK Nanowizard® AFM is ideal for such studies, as the linearization of the JPK Nanowizard® in all three dimensions allows precise imaging (Figure 2) and manipulation of quantum dots (for more information please see our technical report "Nanomanipulation with NanoWizard® technology"). Additionally, use of the JPK
TAO add-on enables the user to align AFM and fluorescent detection to effectively characterise quantum dot optical properties [5]. In this report, however, we shall concentrate on the use of quantum dots in the field of biology.

![Image of quantum dots on glass, imaged in air. In (A) individual quantum dots are visible, whereas in (B) the quantum dots are clustered. The width of the quantum dots is exaggerated due to tip convolution, however the height corresponds to the expected diameter. Height scales: A 0-5 nm, B 0-10 nm.]

**Fig. 2** Quantum dots on glass, imaged in air. In (A) individual quantum dots are visible, whereas in (B) the quantum dots are clustered. The width of the quantum dots is exaggerated due to tip convolution, however the height corresponds to the expected diameter. Height scales: A 0-5 nm, B 0-10 nm.

**Use of quantum dots as a biological label**
Quantum dots have a number of features that make them useful as labels in biological research. Quantum dots exhibit a very sharp emission spectrum. In contrast, chemical and protein fluorophores often exhibit a red tail that can complicate experiments that involve multiple fluorophores of different emission wavelengths. Additionally, as well as a very narrow range of emission wavelengths, a broad range of wavelengths can be used to excite quantum dots. Such properties mean that quantum dots are useful for experiments using multiple fluorophores. Quantum dots also have properties that make them useful for imaging tagged proteins over long time periods, namely, high photostability and a resistance to metabolic degradation. One potential drawback to the use of quantum dots in biological labelling is the fact that, due to their size, they can not diffuse across the cellular membrane. To be introduced into the cell they need to be actively taken up through conjugation to a ligand of a membrane protein that is subsequently internalized, or introduced through microinjection. Additionally, quantum dots blink, and not all quantum dots will light. This can lead to difficulties in labelling all relevant proteins at a surface.

**Experimental Setup**
For these experiments the JPK Nanowizard® AFM was installed on the Olympus IX71 inverted light microscope. Samples were prepared on coverslips and imaged in the JPK Biocell™. The JPK Biocell™ is designed such that high quality optical images (enabled by the thin coverglass) can be obtained simultaneously with high quality AFM (due to the inherent stability of the Biocell™). Optical images of quantum dots were obtained using a CCD cooled to -70°C using circulated water and driven by custom software.

**Fig. 3** Experimental setup. The JPK Nanowizard was installed on an Olympus IX71. Inset shows the view from the front.
Fig. 4 Fluorescence and AFM images of a cell labelled with quantum dots. In panel A, the quantum dot signal has been detected using epifluorescence, panel B is an overlay of two epifluorescent images: one of a marker that labels the whole cell surface and the image from (A) of the quantum dot signal. Panels D-F are IC mode AFM images of dendrites within the area imaged in A and B. All images have been tiled together in panel C. From this overlay of images, the location of quantum dots detected by epifluorescence can be ascribed to specific regions of the AFM image. In H and I (insets 1 and 2 from panel G, respectively) 3D projections of topographic data obtained with AFM are displayed. The single quantum dots are indicated with white arrows, and clusters using yellow arrows, in particular in panel H one can see that there is a cluster of quantum dots.
**Combined imaging of quantum dots**

A combination of quantum dot properties means that the combined imaging of cells labelled with quantum dots using fluorescence and atomic force microscopy can generate unique information. As quantum dots, small though they are, are made of a hard, inorganic substance, they can be distinguished at the surface of cells when imaged using atomic force microscopy. As such, the fluorescent channel can be used to locate quantum dots, and subsequent careful analysis of AFM images can enable determination of whether more than one quantum dot is clustered at a single location, and provide an idea on whether the quantum dot is external to the membrane, or whether it has been internalized.

**Clustered vs single proteins**

When a sample is imaged using atomic force microscopy, contrast is generated on the basis of structural properties. As such, AFM can be used to determine whether single or multiple structures are co-located (as seen in Figure 2). However, in the context of a cell, the quantum dot is so small (approx. 1/1000 of the size) that to identify the location of the quantum dot fluorescent detection is also necessary. In this example, quantum dots were used to label a membrane receptor [6], and an organic fluorescent marker was used to indiscriminately label the surface of the cell, facilitating comparison of AFM images and fluorescent images. Dendrites labelled with quantum dots were then imaged in buffer, using AFM. From the combined images, single quantum dots and small clusters can be identified.

By aligning the various images of the same region of the cell, the location of the quantum dots in the AFM image can be determined. In the height channel acquired using AFM, the quantum dots can be seen, becoming more apparent when the image is displayed as a 3D projection (Figure 4.) In some cases there appears to be a single quantum dot (white arrow), whereas in other cases there is clearly a cluster (yellow arrows).

**Intracellular vs extracellular labelling**

One of the potential limitations of quantum dots is their inability to cross the plasma membrane. However, this means that any intracellular quantum dots must have been internalized in conjunction with the membrane protein that they have been used to label.

A dendritic cell membrane protein was labelled with quantum dots, and after an incubation time known to result in internalization of around 40% of the membrane, protein the cells were fixed. The dendritic cells were then imaged using a combination of bright field, epifluorescence and AFM imaging.

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Fig. 5 Combined images of the same region of a dendritic cell. In (A), a bright-field image of the cell has been overlaid with an epifluorescent image of the quantum dot signal. This is merged with the corresponding AFM image in (B), to allow localisation of the quantum dots. (C) shows an AFM topograph of the relevant region of the cell, with protrusions corresponding to the quantum dot signal circled.
The various images were compiled (Figure 5) and the quantum dot fluorescence was used to identify the location of quantum dots in the AFM image. By taking a cross section through the location of the quantum dot in the AFM image, one can compare the size of the protrusion at that location, and get an indication of the flexibility of the structure. Quantum dots external to the cell are of a size expected for the quantum dots (6nm in the z-direction).

Quantum dots external to the cell can also be seen to move considerably with the scan direction (Figure 6), as the moving tip displaces them. However, internalized quantum dots correspond to protrusions that are much larger than the quantum dots (30-60 nm in the z-direction), and are not displaced during the scanning process. These protrusions likely correspond to subsurface vesicles, into which the labelled membrane protein has been internalized. In some cases there is no protrusion corresponding to quantum dot signal. In this case, the internalized quantum dot is likely no longer in a subsurface vesicle, and as AFM is a surface imaging technique, no longer detected in the AFM image.

Conclusions
The combination of microscopy techniques generates multiple channels of information, as each is used not only to magnify a sample, but to detect particular sample properties based on the contrast mechanism used. In the examples given here, fluorescence was used to detect the location of quantum dot-labelled proteins and then AFM imaging provides additional information about location, and clustering. The design of the JPK Nanowizard® AFM, allowing full integration with light microscopy techniques, means that such a combination of microscopy techniques is now routine. Additionally, the AFM can be combined with high end microscopy techniques such as TIRF, laser scanning confocal and FCS.

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Literature