Comparison of three cell fixation methods for high content analysis assays utilizing quantum dots

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Key words. Cell lines, fixatives, high content screening, nanoparticles, quantum dots.

Summary

Semiconductor nanoparticles or quantum dots are being increasingly utilized as fluorescent probes in cell biology both in live and fixed cell assays. Quantum dots possess an immense potential for use in multiplexing assays that can be run on high content screening analysers. Depending on the nature of the biological target under investigation, experiments are frequently required on cells retaining an intact cell membrane or also on those that have been fixed and permeabilized to expose intracellular antigens. Fixation of cell lines before or after the addition of quantum dots may affect their localization, emission properties and stability. Using a high content analysis platform we perform a quantitative comparative analysis of three common fixation techniques in two different cell lines exposed to carboxylic acid stabilized CdTe quantum dots.

Our study demonstrates that in prefixed and permeabilized cells, quantum dots are readily internalized regardless of cell type, and their intracellular location is primarily determined by the properties of the quantum dots themselves. However, if the fixation procedures are preformed on live cells previously incubated with quantum dots, other important factors have to be considered. The choice of the fixative significantly influences the fluorescent characteristics of the quantum dots. Fixatives, regardless of their chemical nature, negatively affected quantum dots fluorescence intensity. Comparative analysis of gluteraldehyde, methanol and paraformaldehyde demonstrated that 2% paraformaldehyde was the fixative of choice. The presence of protein in the media did not significantly alter the quantum dot fluorescence.

This study indicates that multiplexing assays utilizing quantum dots, despite being a cutting edge tool for high content cell imaging, still require careful consideration of the basic steps in biological sample processing.

Introduction

The use of fluorescent probes for tracking molecules within cells has been a valuable tool in biology. However, conventional probes are prone to photobleaching (Derfus et al., 2004), and have different absorption bands, thereby requiring multiple excitation sources for multiplexing imaging (Chan et al., 2002). The development of semiconductor nanoparticles or quantum dots (QDs) is proving to be an exciting new concept for in vivo, ex vivo and in vitro studies (Dubertret et al., 2002; Wu et al., 2003; Jaiswal et al., 2004). Useful properties include high photobleaching thresholds, long fluorescent lifetimes, broad absorption bands and size controlled emission properties (Alivisatos et al., 2005). Various biomolecules have been conjugated to QDs, e.g. antibodies (Jaiswal et al., 2003), proteins (Clarke et al., 2006; Iyer et al., 2006) and medicinal drugs (Medintz et al., 2003; Byrne et al., 2006). The longer fluorescent lifetimes of these conjugates enables time gating to be preformed, thereby allowing the user to exclude interference from the autofluorescent background (Michalet et al., 2001). Autofluorescence can be one of the major problems of fluorescence microscopy. It is usually caused either by the inherent properties of the biological material, or as an artefact of the fixation process (Baschong et al., 2001).

Alongside the increased use of fluorescent probes, recent years have witnessed a corresponding development of high-throughput imaging and quantitative analysis using automated fluorescent microscopy methods (Pepperkok & Ellenberg, 2006). The presence of an environmentally controlled chamber in some models enables live-cell imaging (Abraham et al., 2004). Due to the ability of QDs to penetrate cellular compartments, it is essential to determine the conditions that optimally conserve their optical properties.
Preservation of biological samples is best achieved by chemical fixation. Fixatives were developed initially to preserve specific cell structures, which could then be visualized by specific staining. Fixation for a short length of time will trap proteins, lipids and carbohydrates in a matrix of insoluble proteins but chemical changes do not occur unless fixation takes place over weeks (Kiernan, 2000). Where fluorescent staining is used, care should be taken that the fixation method does not increase background fluorescence nor enhance any autofluorescence already present. Aldehyde-based fixatives can yield high levels of autofluorescence, due to the presence of unreacted groups remaining in the cells (Collins & Goldsmith, 1981). In assays performed post fixation, it is necessary to ensure that the cells are permeable to probes. Although some fixatives, e.g. methanol, increase the permeability of cells (Harris et al., 1980), others, e.g. paraformaldehyde (PFA), require a permeabilization agent (Heggeness et al., 1977; Melan & Sluder, 1992).

QDs, as inorganic chemical compounds, are susceptible to chemical changes in their environment. Formaldehyde has been suggested for systems incorporating QDs, with varying ranges of concentration and exposure from 1% PFA for 20 min to 2% (10 min) to 4% (20 min) (Jaiswal et al., 2004; Kampani et al., 2007). Some groups have adapted this method to fit in with their particular needs, for example QDs studies involving erythrocytes (Tokumasu & Dvorak, 2003) and in tissue samples (Sukhanova et al., 2002). Other authors have used cold methanol with varying degrees of success (Lidke et al., 2004). Commercial protocols have suggested 2% PFA (Evident Technologies, Troy, NY, USA) or various concentrations of methanol or ethanol on fixing cells prior to the addition of QDs (Invitrogen). It is widely accepted that although gluteraldehyde maintains cell integrity especially for electron microscopy, its suitability when used with fluorescent probes is frequently limited due to high autofluorescence levels (Collins & Goldsmith, 1981).

Any particle introduced into a biological system is likely to become coated with a protein corona (Lynch et al., 2007). As the function of fixatives is to alter the structure of protein, it is possible that any protein coating QDs may also affect the fluorescent properties. Here we present a model of two human cell lines that have been labelled with CdTe QDs possessing different optical properties. We have shown that a medium to low concentration of PFA represents the fixative of choice in studies involving live cells whereas QD fluorescence in systems using prefixed cells is not significantly affected by the nature of the fixative used. However, the nature of the fixative can determine the localization of the QDs.

Materials and methods

CdTe QDs were prepared according to published procedure (Gaponik et al., 2002; Byrne et al., 2006). Various batches of QDs capped with thioglycolic acid (TGA) were used. Stock solutions of nanoparticles were kept in water at 4°C and diluted to a final working concentration of 0.01 mmol immediately before use.

A stock solution of 6% PFA in phosphate buffer saline (PBS) was made and stored at room temperature. A 0.5% solution of Triton™ x 100 (Sigma-Aldrich, Dublin, Ireland) was made up in PBS to permeabilize the PFA fixed cells. One hundred per cent methanol was kept at −20°C until required. Electron microscope grade (35%) gluteraldehyde was used at a final dilution of 1.5% in PBS.

Two different types of cell lines were used, an epithelial cell line, HEp-2. (ECACC, Salisbury, UK) grown in minimum essential medium (Eagle) with Earles Salts (EBSS, Sigma-Aldrich, Dublin, Ireland) and a monocyte cell line, THP-1, (ECACC) grown in RPMI 1640 media. Both types of media were supplemented with 10% foetal calf serum (FCS), 200 mM L-glutamine, 10,000 U/mL penicillin and 10 mg/mL streptomycin. The cells were seeded out into 96-well microtitre plates and onto cover slip slides at a concentration 2 × 10⁵ cells/mL. The THP-1 cells were co-cultured with 100 ng/mL PMA to enable monocyte to macrophage differentiation. Both cell lines were maintained in controlled atmospheric conditions of 37°C, 5% CO₂ until reaching 80% confluence (24 h for HEP-2, 72 h for THP-1). HEP-2 cells were used only in the prefixed cell studies as they have poor phagocytic properties.

After 72 h, media from THP-1 cells was replaced with diluted QDs and then incubated for 3 h at 37°C, 5% CO₂. The cells were then washed, counterstained with Hoechst 33342 (Molecular Probes, Carlsbad, CA, USA), washed twice with media and immediately analysed in controlled atmospheric conditions using the Cellomics KineticScan® (Beckman Coulter. Fullerton, CA, USA). The cells were washed, then fixed with each of the following: 3% PFA, 1.5% gluteraldehyde or 100% methanol and reanalysed. Different concentrations of PFA (1%, 2%, 3%) were also investigated.

The impact of QD exposure to protein was minimized by using serum-free (i.e. no FCS) media, and by direct addition of the QD stock solution. The fluorescent intensity was compared to QDs pre-diluted in complete media and exposed to cells also in complete media.

Cells, to be fixed prior to the addition of QDs, were allowed to reach 80% confluence, then washed twice with PBS at room temperature and subsequently fixed by either of the methods described below.

In the first series of experiments the cells were treated with 3% PFA for 30 min. washed again and then permeabilized with 0.5% Triton® x 100 for 15 min. These were washed twice with PBS and then left in PBS. The plates were then sealed with parafilm and kept at 4°C until required.

Alternatively the cells were fixed for 10 min in cold (−20°C) methanol, allowed to dry, sealed with parafilm and stored at −20°C until required.
Diluted QDs were added to THP-1 and HEp-2 cells and then incubated for 1 h. The plates were washed twice in PBS, stained with Hoescht for 3 min, washed again with PBS and left in PBS to be analysed using a Cellomics KineticScan® (KSR). All assays throughout the experiments were performed in triplicate.

The images from the microtitre plates were acquired using the KSR® and analysed on the Cellomics Toolbox Scan® with the Compartmental Analysis® bioapplications. Filters are allocated to different channels to detect specific emission wavelengths. A user-defined gate is applied to the nucleus (as indicated by Hoechst staining) in channel 1 [\(\lambda_{\text{ex}} 360 \pm 50\), \(\lambda_{\text{em}} 515 \pm 20\) nm] that identifies the areas relating to the nucleus and cytoplasm in channel 2 [\(\lambda_{\text{ex}} 475 \pm 40\), \(\lambda_{\text{em}} 515 \pm 20\) nm] and channel 3 [\(\lambda_{\text{ex}} 560 \pm 15\), \(\lambda_{\text{em}} 600 \pm 25\) nm]. Information can then be obtained on parameters such as number, size, shape and fluorescent intensities on the cells of interest (Fig. 1a). Organelles, which are shown as discreet fluorescent inclusions within the cytoplasm or nucleus can also be identified (Fig. 1b).

In order to confirm effects of fixatives upon stability and luminescence of the QDs, TGA stabilized CdTe (1.4 \(\times\) 10\(^{-5}\) M, 200 \(\mu\)L) (emission in 600 nm range) was added to RPMI 1640 culture medium (1800 \(\mu\)L). This solution was added to a 1 cm path length fluorimeter cuvette. All emission spectra were recorded on a Varian Cary Eclipse spectrophotometer (Varian Cary, Lake Forest, CA, USA). The emission of this sample was recorded, using excitation wavelength of 560 nm. Then, 20 \(\mu\)L aliquots of PFA (6%) were added to the sample and the emission spectrum was recorded after each addition. The total volume of PFA added was 200 \(\mu\)L.

**Results and discussion**

The TGA capped CdTe QDs were taken up by the live phagocytic THP-1 cells and localized within the endosomes/lysosomes in the cytoplasm (Fig. 2a–c). They had emissions in the range of 600 nm and therefore were detected as red fluorescence in channel 3 on the KSR. As they did not appear to enter the nuclei, all analysis was carried out on the cytoplasm only.

Comparing the fluorescence intensity of QDs in living cells, those fixed with methanol or 3% PFA showed a decrease in intensity of staining. This was confirmed by spectrophotometry that showed quenching of QD luminescence with increasing quantities of PFA (Fig. 3). Methanol fixation caused a dramatic 10- to 100-fold reduction in the intensity of the fluorescent signal from the QDs, which may be explained by the instability of QDs in a hydrophobic environment.

We also investigated glutaraldehyde as an alternative fixative for use with the QDs due to its excellent structure preserving properties, particularly for electron microscope. However, we found very high levels of autofluorescence in all channels on the KSR extending up to emission wavelengths of 720 nm (Fig. 4). Therefore, we can conclude that glutaraldehyde is unacceptable for High Content Screening (HCS) studies incorporating QDs.

As we found that 3% PFA fixation significantly reduced fluorescence intensity when compared to live cells, we proceeded to examine lower concentrations of PFA.

Changes in nuclear size and in the intensity of Hoescht staining are indicative of the cell’s integrity being affected
Fig. 2. Comparison of fluorescent intensities using different fixatives in THP-1 cells. The intensity of quantum dot (QD) fluorescence is shown in (a) live cells, (b) cells treated with 3% PFA and with (c) methanol fixation. Quantitative differences are shown (d) for cells without QDs (white bars) and with QDs (striped bars). Each bar represents triplicates of a single batch of QDs. RFU: relative fluorescent units. Results shown as mean and standard error of the mean.

Fig. 3. Photoluminescence of thioglycolic acid (TGA) capped quantum dots (QDs). Effect of PFA on emission spectrum of TGA CdTe. This spectrum shows quenching of QD luminescence with increasing quantity of PFA.

by variation in fixative concentration. 1% PFA fixation caused a notable reduction in nuclear size in contrast to 2% and 3% solutions which both equally maintained the nuclear integrity (see Fig. 5a and b). On the other hand, the intensity QD fluorescence emission was preserved with reduced concentrations of fixative. As fixation by 2% PFA resulted in better preservation of QD fluorescence emission than 3% PFA and at the same time had no adverse affects on cell integrity, it was selected as the concentration of choice (Fig. 5c). Time course studies were also performed and 20 min was found to be the optimum incubation time for 2% PFA fixation (data not shown).

We also show that minimizing QD exposure to protein prior to incubation with the cells made no difference to QD fluorescent intensity. However, incubating the QDs in cells with complete media does show a small enhancement of QD uptake (Fig. 6). As this study indicates that pre-diluting QDs in serum-free media is not required for their optimal uptake, the number of manipulations can be decreased in biological assays utilizing QDs. This feature would also be beneficial for future in vivo applications (Morris et al., 2001).

Smaller TGA capped QDs were used for the prefixed assay, emitting in the region of 515 nm and therefore detectable in channel 2. These QDs localized within the cytoplasm...
Fig. 4. Glutaraldehyde-induced autofluorescence in THP-1 cells. THP-1 cells were fixed with either glutaraldehyde (a, b, c, d) or 2% paraformaldehyde (e, f, g, h). Images show autofluorescence (a, e) with excitation 475(40), emission 515(20), green; (b, f) excitation 560(15), emission 600(25), red; (c, g) excitation 655(30), emission 730(50), far red and (d, h) composite. The nuclei are stained with Hoescht (blue). Scale bar 10 mm.
Fig. 5. Effects of various concentrations of PFA on nuclear size, staining intensity and quantum dots (QDs) fluorescence emission. Changes in nuclear size (a), nuclear intensity (b) and QDs fluorescence emission intensity (c) in THP-1 cells, after fixation with different PFA concentrations. White bars reflect the data from cells without QDs and dark bars from those exposed to QDs. Final concentration of QDs was ∼0.01 mM. RFU: relative fluorescent units. Results shown as mean and standard error of the mean.

Fig. 6. Impact of quantum dot (QD) exposure to protein. Variations due to the presence or absence of protein in the medium, and whether or not the QDs were added directly from stock (grey bars) or pre-diluted (black bars) prior to addition to the cells are also shown. Cells without QDs are indicated as white bars. Concentration of QDs was 0.01 mM. RFU: relative fluorescent units. Results shown as mean and standard error of the mean.

of both the PFA (not shown) and methanol fixed cells [Fig. 7a(i) and b(i)]. They may bind to cytoskeletal structures within the cytoplasm. When the PFA fixed cells were permeabilized with Triton ×100 the QDs appeared in the nuclei localizing within the nucleoli [Fig. 7a(ii) and b(ii)]. Although fixing HEp-2 and THP-1 cells with methanol prior to the addition of QDs preserved the cells in perfect condition, the QDs remained distributed only in the cytoplasm. QDs in the permeabilized PFA fixed cells located in the nucleoli of both cell types especially in the THP-1 cells. This was shown quantitatively with the KSR where the intensity of THP-1 nucleoli staining was 2000 RFU (relative fluorescent units), compared to 1500 RFU in the nuclei and 600 RFU in the cytoplasm (Fig. 7c).

Similarly, the nucleoli of the Hep-2 cells were of higher intensity (1500 RFU) than the nucleus (1200 RFU); however, the difference were not as marked and there was little change in fluorescence intensity comparing the nucleus and cytoplasm (Fig. 7d). This is probably due the diverse functionality of the two cell types.

The fixation of cells containing QDs will continue to play an important part in any in vitro experiments. In this report we have only examined the effects that fixation has on the QD fluorescent intensity within the cells. Other authors have noted various phenomena that can be attributed to fixatives, including QDs that form nanowires in the presence of methanol (Sukhanova et al., 2007) or fixatives that increase the uptake of particles (Richard et al., 2003). Unlike some reports (Voura et al., 2004) we have found that PFA can have an adverse affect on the intensity of staining if added post incubation, but that this can be influenced by the physicochemical properties of the QDs. Methanol fixation of cells containing QDs dampened the signal significantly and cannot be recommended as a fixative in live cell analysis. Gluteraldehyde enhances autofluorescence especially in the 550–700 nm region of the spectrum, and therefore should not be used as many QDs emit in this area.
Factors affecting quantum yield of the QDs include pH, hydrophobicity and ionic charge (Parak et al., 2003). Therefore, fixing cells prior to the addition of the QDs should avoid the adverse affect on the quality of the resultant fluorescence. Our studies verify this, and also indicate that as the localization of the QDs within the cell can be fixation dependent, care must be taken when choosing the appropriate fixative. It has previously been shown that the type of fixative can influence the location of proteins within the cell causing them to move from the cytoplasm to the nuclei and even in some cases the nucleoli (Melan & Sluder, 1992). A similar process probably is occurring with QDs.

This report emphasizes the need to optimize fixation techniques when setting up any assay involving nanoparticles.

Acknowledgements

This project was in part funded by the Health Research Board, NanoInteract FP-6 Consortium and BioNanoInteract Science Foundation of Ireland Research Cluster.

References


