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Quantum dots as new-generation fluorochromes for FISH: an appraisal

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Abstract In the field of nanotechnology, quantum dots (QDs) are a novel class of inorganic fluorochromes composed of nanometre-scale crystals made of a semiconductor material. Given the remarkable optical properties that they possess, they have been proposed as an ideal material for use in fluorescent in-situ hybridization (FISH). That is, they are resistant to photobleaching and they excite at a wide range of wavelengths but emit light in a very narrow band that can be controlled by particle size and thus have the potential for multiplexing experiments. The principal aim of this

study was to compare the potential of QDs against traditional organic fluorochromes in both indirect (i.e. QD-conjugated streptavidin) and direct (i.e. synthesis of QD-labelled FISH probes) detection methods. In general, the indirect experiments met with a degree of success, with FISH applications demonstrated for chromosome painting, BAC mapping and use of oligonucleotide probes on human and avian chromosomes/nuclei. Many of the reported properties of QDs (e.g. brightness, ‘blinking’ and resistance to photobleaching) were observed. On the other hand, signals were more frequently observed where the chromatin was less condensed (e.g. around the periphery of the chromosome or in the interphase nucleus) and significant bleed-through to other filters was apparent (despite the reported narrow emission spectra). Most importantly, experimental success was intermittent (sometimes even in identical, parallel experiments) making attempts to improve reliability difficult. Experimentation with direct labelling showed evidence of the generation of QD-DNA constructs but no successful FISH experiments. We conclude that QDs are not, in their current form, suitable materials for FISH because of the lack of reproducibility of the experiments; we speculate why this might be the case and look forward to the possibility of nanotechnology forming the basis of future molecular cytogenetic applications.

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Abbreviations

BAC(s)	bacterial artificial chromosome(s)
BSA	bovine serum albumin
DAPI	4',6-diamidino-2-phenylindole
ddH ₂ O	double-distilled water
DS	dextran sulfate
DOP	degenerate oligo primed
DTT	dithiothreitol
dUTP	2'-deoxyuridine 5'-triphosphate
FA	formamide
FISH	fluorescence in-situ hybridization
FITC	fluorescein isothiocyanate
HFEA	human fertilization and embryology authority
MAA	mercaptoacetic acid
NIR	near infrared
PBS	phosphate-buffered saline
QD	quantum dot
QD-FISH	quantum dot fluorescence in-situ hybridization
RT	room temperature
PCR	polymerase chain reaction
SERT	serotonin transporter protein
SSC	saline sodium citrate
UV	ultraviolet

Introduction

Traditionally associated with engineering and physical science (e.g. in computer chips), 'nanotechnology' is a research field that manipulates and creates structures of particles with dimensions smaller than 100 nm (Chan 2006). Within the last decade, however, there has been a growing interaction between nanotechnology and biology (Parak et al. 2003), particularly in fluorescence microscopy. One novel class of inorganic fluorophores arising from nanotechnology and useful in fluorescent microscopy are 'quantum dots' (QDs) (Miller and Chemla 1986; Reed et al. 1986). QDs are composed of nanocrystals of a semiconductor material (e.g. either cadmium sulfide (CdS), cadmium selenide (CdSe), indium phosphate (InP) or lead selenide (PbSe)) at the core (Lipovskii et al. 1997). This is coated with a (usually zinc sulfide, ZnS) shell that improves the optical properties (Michalet et al. 2005; Invitrogen 2006); plus an extra polymer coating that

serves as a site for conjugation with biomolecule moieties. This brings the total size of the nanocrystal to 10–20 nm. The core material is chosen according to the emission wavelength range that is targeted (e.g. CdS for ultraviolet-blue, CdSe for the visible spectrum and CdTe for the far red and near infrared (Quantum Dot Corporation 2006); thus fluorophore colour is size-dependent and controlled during synthesis (Chan et al. 2002).

A unique property of QDs is their broad excitation and narrow symmetric emission spectra. The full spectral width of QDs at half maximum is 12 nm and leads to less overlap between absorption and emission spectra (Chan and Nie 1998). Thus different QDs can be excited by a single wavelength shorter than their emission wavelength (Green 2004; Alivisatos et al. 2005; Arya et al. 2005). Such an approach cannot be achieved with classical organic fluorophores because they have narrow excitation and broad emission that often results in spectrum overlap or red tailing (Dabbousi et al. 1997). QDs produce significantly brighter fluorescence (2–11 times) (Larson et al. 2003) because of the large molar extinction coefficients (10–50 times larger than those of organic fluorophores) (Gao et al. 2005). Due to their inorganic composition they are more resistant to photobleaching than organic fluorophores (Alivisatos 1996; Bruchez et al. 1998; Michalet et al. 2001; Jaiswal et al. 2003; Parak et al. 2005) and have a longer fluorescence half-life than typical organic dyes (Lounis et al. 2000).

There are many *in vitro* applications using QDs reported in the literature. For instance: detection of the cancer marker Her2 on the surface of fixed and live cancer cells (Wu et al. 2003), targeting the serotonin transporter protein (SERT) in transfected HeLa cells and oocytes (Rosenthal et al. 2002), and identifying the erbB/HER family of transmembrane receptor tyrosine kinases that mediate cellular responses to epidermal growth factor (Lidke et al. 2004). QDs have been used as cellular markers because they can be internalized by cells using a receptor (Chan and Nie 1998; Zheng et al. 2006) or by non-specific endocytosis (Parak et al. 2002). QD cell markers have been used in cell–cell interaction studies by creating unique colour tags for individual cell lines (Mattheakis et al. 2004). In addition, QD resistance to photobleaching has enabled 3D optical sectioning studies of the vascular endothelium

(Ferrara et al. 2006), applications in cell motility assays for studying actomyosin function (Mansson et al. 2004), and phagokinetic tracking of small epithelial cells responsible for 90% of cancers (Parak et al. 2002).

The optical properties of QDs have also been exploited for *in vivo* uses. For instance, as a means to deliver drugs to target molecule sites after injection (Akerman et al. 2002) and to study the behaviour of specific cells during early stage embryogenesis in *Xenopus* and Zebrafish embryos by microinjection of micelle-encapsulated QDs (Dubertret et al. 2002; Rieger et al. 2005). Gao et al. (2004) reported *in vivo* cancer targeting and imaging using antibody-conjugated QDs for human prostate cancer and QDs have been used as contrast agents during surgery to map sentinel lymph nodes in the pig and the mouse (Kim et al. 2004).

Given the potentially much-vaunted properties of QDs, they seem as ideal candidates for the study of chromosomes through adaptations of FISH protocols. Since its inception, FISH has continuously evolved but, as with all experiments involved in fluorescent microscopy, faces limitations imposed from the use of organic fluorophores. The number of available fluorochromes and their broad emission spectra make multicolour experiments difficult to resolve due to overlapping and the rapid photobleaching of organic fluorochromes. Published work related to QD-FISH is currently limited. Xiao and Barker (2004b) utilized biotinylated total genomic DNA on human metaphase chromosomes detected using streptavidin-conjugated QDs. Comparisons between detection with QDs and organic fluorochromes (Texas Red–streptavidin and FITC–streptavidin) showed that QD probes were significantly more photostable and 2–11 times brighter than organic fluorochromes. Furthermore, they applied this technique to detect the Her2 locus in low-copy human breast cancer cells, demonstrating that QD-FISH has the potential to become a medical diagnostic tool. A similar indirect labelling approach has been used on plant chromosomes (Muller et al. 2006) with limited success. Chan et al. (2005) developed a direct labelling approach to target specific mRNAs in mouse brain sections. Biotinylated labelled oligonucleotides were conjugated with QD–streptavidin in the presence of biocytin to block excess streptavidin sites that could result in oligonucleotide cross-linking. Bentolila and Weiss (2006)

using a biotin–streptavidin strategy, labelled oligonucleotide probes with QDs; in this case complexes were analysed using gel electrophoresis and the optimum molar ratio of QD–DNA was used against the major (γ) family of mouse satellite DNA in both interphase and metaphase preparations. In addition they also used oligonucleotides labelled with different coloured QDs to target two classes of repetitive DNA in the centromeric region. Their results showed that QD-based probes are more efficient at hybridization than organic fluorochromes and have great potential in multicolour assays. Furthermore, Jiang et al. (2007) generated QD–genomic DNA probes to visualize gene amplification in lung cancer cells, while the most recent study involving direct labelling of maize chromosomes was published by Ma et al. (2008), in which QDs were solubilized with an MAA (mercaptoacetic acid) monolayer and then a thiol–DNA to create probes. Apparently, with this method, the probes were small enough to hybridize with the DNA sequences. This study also highlights the problem of steric hindrance regarding QDs and that pH (Xiao et al. 2005), ionic strength and formamide (FA) could affect the affinity of QD–probes for chromosomal targets (Ma et al. 2008).

Given the potential of QD-FISH, it is puzzling how few studies (notwithstanding the above) there are in this area. Clearly more studies are required to explore the use of QD-FISH. For instance, we are aware of no published data using QD-labelled probes to target whole chromosomes (chromosome painting) either in two dimensions or in 3D nuclear organization studies. The overall aim of this study was to therefore to explore the use QDs in the place of organic fluorochromes, specifically with a view to using QDs in multiplex experiments (i.e. to target multiple regions simultaneously).

The specific aims of the current study were thus as follows: (a) to ask whether streptavidin–QD conjugates could be used for the detection of biotinylated (or digoxigenin) labelled probes in ‘indirect’ FISH labelling experiments under a range of conditions; and (b) to develop strategies for the direct coupling of QDs to biotinylated probes (including oligonucleotides and chromosome paints) for use in ‘direct’ FISH experiments (with the ultimate goal of performing multiplex experiments).

Materials and methods

Biological material

Lymphocytes from peripheral blood cultures and sperm from freshly ejaculated semen samples formed the basis of target material for most of the experiments. Both cell types were obtained after written consent from a chromosomally normal male donor. Research was approved by the Research Ethics Committees of the University of Kent and carried out under the auspices of the treatment licence awarded by the Human Fertilization and Embryology Authority (HFEA). Whole blood was cultured in PB Max™ Karyotyping Medium (12557-013 Gibco/BRL, Invitrogen UK) arrested in metaphase using colcemid (D1925, Sigma, St Louis, MO, USA) then swelled and fixed to glass slides using 75 mM KCl and three changes of 3:1 methanol–acetic acid. Fresh ejaculate was washed in 10 mM NaCl/10 mM Tris pH 7.0 sperm wash buffer and then centrifuged for 7 min at 1900 rpm. The supernatant was removed and resuspended up to 5 times depending on the pellet size and colour. The sample was then fixed in a drop-wise fashion using 3:1 methanol–acetic acid to final volume of 5 ml. The process was repeated up to 5 times (pellet dependent) and 5–20 µl of the sample was spread on a poly-L-lysine-coated slide (631-0107, VWR, West Chester, PA, USA) (for better fixation of cells) and air dried at room temperature (RT). In addition, cultured embryonic fibroblasts from chicken and turkey were used; cells were suspended in metaphase using colcemid, trypsinized, swelled and fixed for cytogenetic analysis by standard protocols. For all experiments performed with avian samples or human lymphocytes, superfrost glass slides (AG00008232E, Menzel-Glaser, Braunschweig, Germany) were used.

QD-streptavidin conjugates

Two suppliers were used for these experiments, Invitrogen, Carlsbad, CA, USA (QD525 and QD585) and Evident Technologies, Troy, NY, USA (QD520, QD600 and QD620).

Source of probes

In early experiments, a commercially available pan-centromeric probe (1695-B-02, Cambio, Cambridge,

UK) was utilized, as were bacterial artificial chromosomes (BACs) from chicken labelled with biotin by nick translation. Also, in-house chromosome paints were generated from flow-sorted human and chicken chromosomes (a kind gift from the Department of Pathology, University of Cambridge). The degenerate primer 6MW (5'→ 3' CCG ACT CGA GNNN NNN ATG TGG) was used in a standard DOP-PCR experiment to generate sufficient material, which was then labelled with biotin or digoxigenin via nick translation and used in indirect FISH experiments. A custom-made DOP-PCR primer labelled with biotin (through a C6 linker; Invitrogen, personal communication 2009) was used to generate DOP-PCR products with a single biotin on each length of DNA for direct QD conjugation experiments (Invitrogen). In addition, for direct labelling experiments (and for indirect FISH), an oligonucleotide probe specific for a region on chromosome 12 with a single biotin molecule attached to the 5' end was used. The biotin was incorporated during synthesis through biotin phosphoramidite by linking the 5' OH to the phosphorus atom (Sigma Genosys, personal communication 2009).

The following protocol (Bentolila and Weiss 2006) was used to couple streptavidin-conjugated QDs to biotinylated oligonucleotides and chromosome paints labelled with a single biotin molecule. Direct coupling requires probes to have a single biotin (per primer binding site) to prevent QD aggregation and therefore unspecific signals. PCR products were purified using a QIAquick spin column (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. QD:DNA constructs (i.e. FISH probes labelled with QDs) were made by mixing 1 µl of 500 nM QD with 1 µl of 50 ng/µl biotinylated probe. These were gently vortexed for 5 s, allowed to incubate at room temperature for a minimum of 30 min and stored on ice until ready for use. The QD:DNA construct was purified (from unbound probe) using S300 columns (GE Healthcare UK S-300 HR) following the manufacturer's instructions. In order to establish that the QD-DNA complex still had fluorescent activity, the tube was checked for fluorescence under a UV transilluminator. To test for QD:DNA construct formation, standard 2% agarose gel electrophoresis was used under the premise that 'naked' DNA has greater mobility than QD-conjugated DNA and than QD alone.

For all experiments, 100–200 ng/ μ l of probe was dissolved in standard hybridization buffer (50% formamide (20% for oligonucleotide probe), 2 \times SSC, 10% dextran sulfate, 60–200 μ g of salmon sperm DNA). For direct FISH experiments, formamide was reduced to 25%, dextran sulfate was removed, and 5 \times Denhardt's solution together with 50 mM phosphate buffer, 1 mM EDTA were included. For the commercial pancentromeric probe, the manufacturer's standard hybridization buffer was used and the probe was denatured at 85°C prior to use according to the manufacturer's guidelines.

FISH

Slides containing metaphase preparations were dehydrated in an ethanol series, air dried and treated with 100 μ g/ml RNase under a coverslip (Menzel-Glaser) at 37°C for 1 h, then washed twice in 2 \times SSC for 5 min each, before a second ethanol series and air drying. Slides bearing sperm preparations were washed in 0.1% DTT, 0.1% Tris-HCl (pH 8.0) at room temperature for 20–30 min to swell the sperm heads and then rinsed in 2 \times SSC. This was followed by pepsin treatment in a pre-warmed at 39°C Coplin jar with 49 ml of ddH₂O, 0.5 ml of 1 N HCl, 0.5 ml of 1% pepsin for 20 min. Slides were subsequently washed in ddH₂O followed by rinsing in 1 \times PBS before incubation in 4% paraformaldehyde/PBS (pH 7.0) at 4°C for 10 min; slides were then rinsed with 1 \times PBS followed by ddH₂O at room temperature and another ethanol series was carried out at RT for 2 min each and slides were air dried.

The cells were then denatured at 70°C in 70% formamide/2 \times SSC (pH 7.0) for 2 min (8–10 min for sperm) before washing with 70% ice-cold ethanol for 2 min followed by 80% and 100% ethanol for 2 min each prior to air drying.

Labelled probe in hybridization buffer (10 μ l) was denatured at 65–85°C for 1–10 min, then added to a specified marked area under a 18 \times 18 mm coverslip, which was sealed with rubber cement and hybridized at 37°C overnight. For direct labelling experiments, the slides were heated at 80°C for 3 min to prevent any reannealing of the DNA strand after denaturation. The rubber cement was removed and slides were washed in 2 \times SSC to remove the coverslips. Slides were then washed in 37°C 50% formamide–2 \times SSC solution for 20 min (2 \times 5 min in 20% formamide–2 \times

SSC solution at 37°C for oligonucleotide probes), then for 1 min in 2 \times SSC, 0.1% Igepal (v/v) at RT. For indirect FISH, slides were incubated in storage buffer (4 \times SSC, 0.05% Igepal (v/v)) for 15 min, then in blocking buffer (4 \times SSC, 0.05% Igepal (v/v), 3% BSA (w/v)) for 25 min at RT. The detection mix (QD-conjugated streptavidin for experiments and Cy3-conjugated streptavidin for controls) was prepared at 4°C for 20–25 min before use, centrifuged at 1300 rpm for 5 min, then applied to the slide under coverslip and incubated for 35 min at 37°C. For QD conjugates the detection mix consisted of 1 μ l of QD in 99 μ l of TNB buffer (pH 7.5) (0.1 M Tris-HCl, 0.15 M NaCl, 0.5% BSA (w/v)) per slide; for controls, the detection mix was Cy3-streptavidin in blocking buffer diluted 1:200. The coverslip was then removed and slides were washed in fresh storage buffer (in the dark) for 10 min, followed by a brief rinse with ddH₂O. Slides were then air-dried and counterstained using Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). Direct FISH experiments had post-hybridization washes of 2 \times 10 min in TST buffer (0.1 M Tris, 0.15 M NaCl, 0.05% Tween 20 (v/v), 2 \times SSC pH 7) at 37°C then proceeded straight to the ddH₂O stage following post-hybridization washes.

Variations to protocol

In order to improve the efficacy and reliability of the QD experiments, various FISH conditions were altered, including removal of the block buffer step and changing the temperature and time of the post-hybridization washes.

To test the hypothesis that the presence or absence of dextran sulfate in the hybridization mix affected subsequent binding of QD conjugates in indirect FISH experiments (the direct QD FISH hybridization mix did not contain dextran sulfate), controlled experiments with and without dextran sulfate in the hybridization mix were performed.

To minimize steric hindrance of the biotin, biotin-21-dUTP was used in place of biotin-16-dUTP in both direct and indirect experiments. Also, the effects of different ratios of biotin labelled and unlabelled probes were assessed to minimize steric hindrance.

To determine whether there was a hapten-specific effect (i.e. whether biotin per se, was the best hapten to use) we attempted to detect digoxigenin-

labelled probes with mouse anti-digoxigenin antibody followed by a layer of QD-conjugated goat anti-mouse antibody.

To test the hypothesis that QD conjugates were aggregating and adhering to the sides of the tube, we performed controlled experiments sonicating the conjugates before use and using siliconized tubes and pipette tips.

To test the hypothesis that use of DAPI as a counterstain could affect visualization of the QDs, experiments were performed with and without DAPI.

Results

Indirect labelling

Use of streptavidin-conjugated QD525 and QD585 produced a degree of success in generating analysable preparations for FISH experiments. Figures 1, 2, 3, 4, 5 and 6 demonstrate successful experiments (some compared with Cy3 controls). We were successful in hybridizing chromosome paints from both human and birds to metaphases and interphases of the same species (Figs. 1, 2, 3 and 4); BAC clones for chicken chromosomes successfully hybridized (Fig. 5); and the oligonucleotide sequence specific for chromosome 12 gave a reproducible signal (Fig. 6).

By and large, when results were successful, the properties of QDs were apparent. Most notably, the

preparations were significantly brighter by visual inspection than Cy3 preparations and were resistant to photobleaching. That is, when Cy3-labelled preparations were exposed continuously to light, photobleaching occurred after about 5 min. On the other hand, when QD preparations were exposed to light, no appreciable loss of signal was seen after one hour of exposure.

We also observed that preparations displayed the phenomenon known as ‘blinking’; that is, when samples were visualized the fluorescent signal repeatedly appeared to switch ‘on and off’. In general terms, QD preparations in these experiments had more background than was observed for Cy3 preparations. Also, there was a notable difference in the appearance in the fluorescent signal from QD compared to Cy3, which is perhaps best explained with an analogy: Cy3 signals gave the impression of examining fluorescent ‘dust’ compared the fluorescent ‘rocks’ impression given by the QDs. It was noticeable that, in many chromosome painting experiments, the QD signal was brighter around the periphery of the chromosome, giving the impression of a fluorescent ‘sheath’ (Fig. 3); moreover, in selected cases, a bright signal was visible in the interphases of the cell but not the metaphases. Another point of note was that the emission spectra of the QDs did not appear to be as narrow as the manufacturers claimed. That is, despite the use of narrow band-pass filters, QD525 and QD585 each showed a significant ‘bleed-through’ into the channel of the other. Most importantly, however, it was noticeable that, while the Cy3

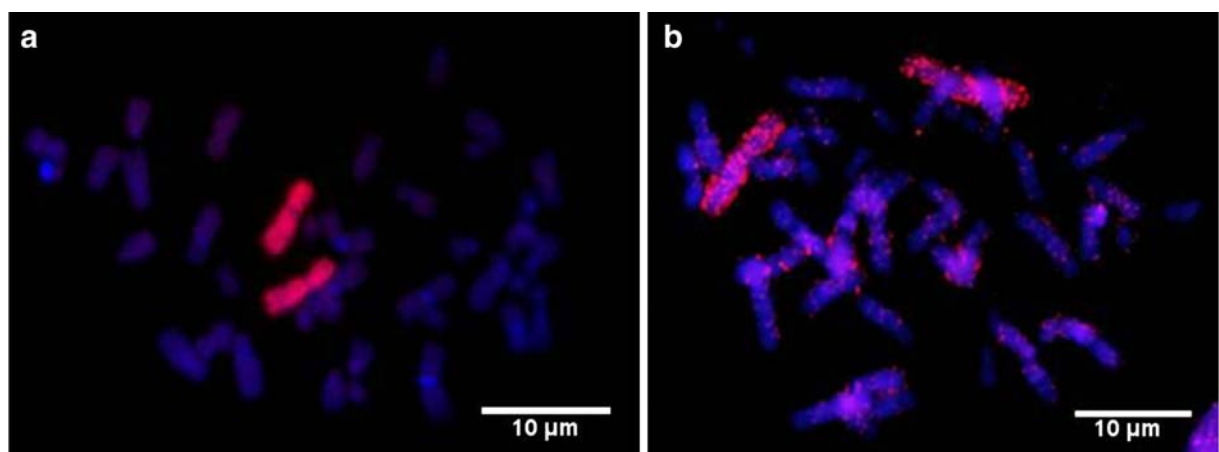


Fig. 1 Detection of biotinylated human chromosome paint 2 with **a** Cy3-conjugated streptavidin; **b** QD585-conjugated streptavidin. The Cy3-labelled probe gives a more specific signal with less background

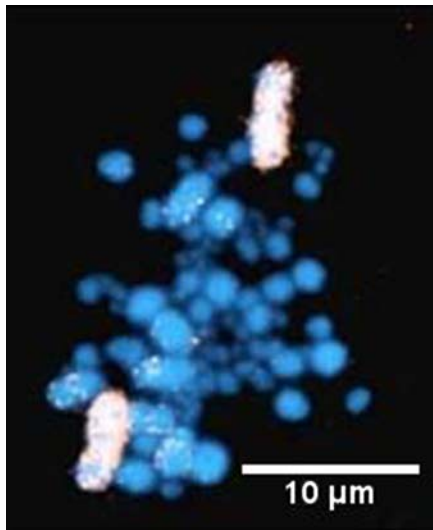


Fig. 2 FISH of turkey chromosome 1 paint to turkey chromosomes using QD525-conjugated streptavidin

controls worked successfully with rare exceptions, success from equivalent QD experiments was notably intermittent. In particular identical QD experiments could often be perfectly successful on one day but unsuccessful on the next or, even more confusingly, identical experiments run in parallel would work for one slide but not the other on a regular basis. As an overall estimate, indirect QD experiments were successful 25–35% of the time when controls gave an acceptable result (>95%).

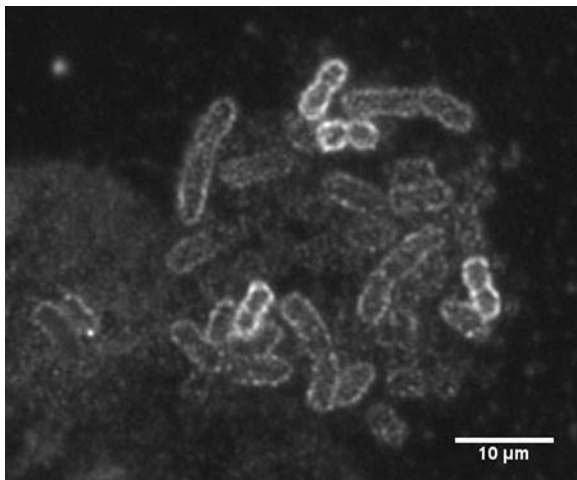


Fig. 3 FISH of chicken chromosome 2 paint to a chicken tetraploid chicken metaphase using QD525-conjugated streptavidin. Hybridization signals are brighter at the periphery of chicken chromosome 2 where the chromatin is less condensed

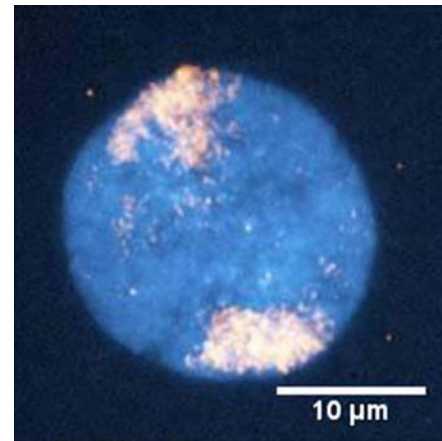


Fig. 4 Turkey nucleus showing hybridization of turkey chromosome 4 paint detected by QD525-conjugated streptavidin

In general terms, amidst this background of intermittent success, we were unable to identify any particular factor that would improve the success of the experiments. Controlled studies varying hybridization times and temperatures did not especially favour QD experiments on any occasion. There was no appreciable difference whether or not the blocking buffer and/or dextran sulfate in the hybridization mix and/or

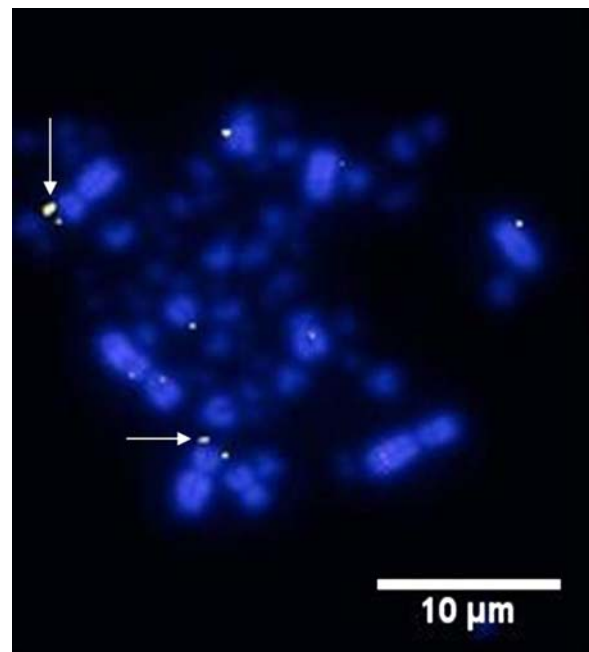


Fig. 5 Hybridization of a BAC probe to terminal chromosome 2p in chicken using QD525-conjugated streptavidin. Arrowheads indicate the specific hybridization sites (2p)

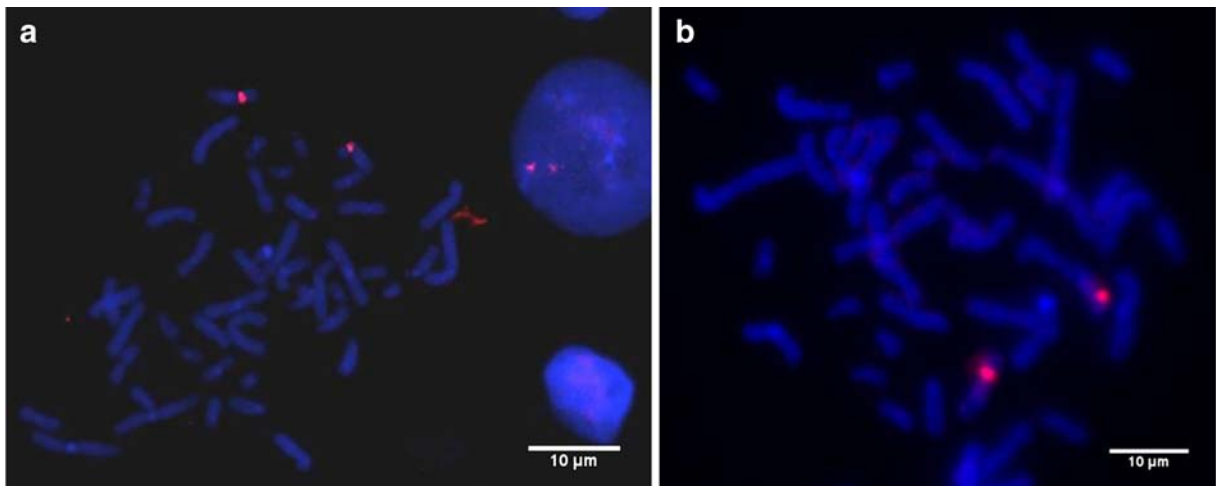


Fig. 6 FISH hybridization of an oligonucleotide probe for the centromere of human chromosome 12 on human metaphases detected by **a** QD585-conjugated streptavidin; **b** Cy3-conjugated streptavidin

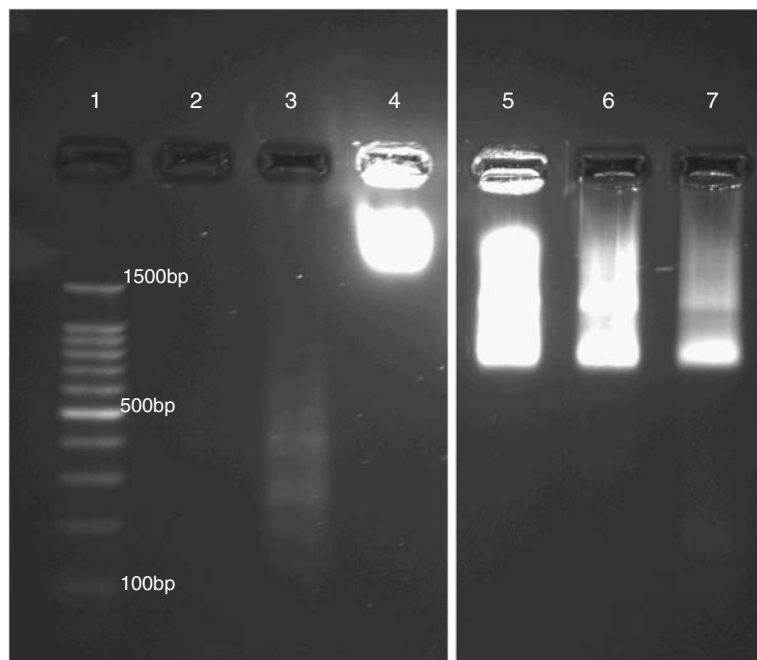
DAPI in the mountant was used. We did observe good signals through the use of biotin-21-dUTP; however, this was, at least by visual inspection by a number of observers, not noticeably different from the use of biotin-16-dUTP, nor did our efforts to vary the relative concentrations of labelled versus unlabelled probes allow us to draw firm conclusions. The only intervention that we observed to demonstrate a degree of success was the use of silicon-coated Eppendorf

tubes and sonication of the conjugate prior to use. In both scenarios we observed an improvement (albeit temporary) in the reliability of the results.

Direct FISH

Efforts to conjugate streptavidin-QDs to biotinylated DNA were initially encouraging. Figure 7 demonstrates a noticeable shift in the mobility of the DNA-QD

Fig. 7 Agarose gel (selected lanes from the same gel) showing differential motility of amplified biotinylated DNA (lane 3), QD alone (lane 4), and QD:DNA construct at varying concentrations (lanes 5–7). The differential motility seen in lanes 5–7 indicates that the construct was successfully generated. Lane 1 is a 100 bp ladder and lane 2 is blank



construct compared with either biotinylated DNA alone or streptavidin QD alone. These results were reproduced on approximately 20 occasions for both the oligonucleotide chromosome 12 probe and the chromosome paints; however, repeated attempts at subsequent FISH experiments (employing a range of different conditions of stringency, hybridization buffer, etc.) without exception ended in failure (despite known Cy3 conjugate controls working reliably).

Finally, it is worth noting that records from all QDs purchased were kept and results were obtained only through the use of Invitrogen samples (Lot 48184A, for QD585). In contrast, there were no results through the use of Evident samples.

Discussion

To the best of our knowledge, this is the first study to demonstrate a comprehensive appraisal of the utility of QDs for FISH experimentation. That is, while several studies have demonstrated the use of QDs in FISH, as with the majority of studies in the literature, there may be a tendency to present only the positive data. QD-based FISH studies are conspicuous mostly by their absence (Xiao and Barker 2004a; Bentolila and Weiss 2006; Ma et al. 2008); that is, if QDs had fulfilled their promise they would, at least in part, have replaced organic fluorochromes. One would expect orders of magnitude more QD-FISH papers in the literature and several companies marketing QD-labelled probes, which—at the time of writing—is simply not the case.

While we would not claim that we have explored every possible avenue with respect to QD-FISH, we have extensive experience in FISH over many years and have, for the last three or four of them, been running parallel QD-based experiments, mostly in avian and human cells. Put simply, lack of reproducibility appears to be the hallmark of QD-FISH in contrast to the more robust applications with antibody conjugates for cell labelling. This is possibly because of incomplete technical knowledge of the factors associated with penetration of a QD probe into a complex structure such as a chromosome or nucleus. Furthermore, in commercially available QD-streptavidin conjugates we are yet to understand many chemical and physical factors that are well understood for organic fluorophore conjugates (e.g. FITC, Texas red and the Cy dyes).

For these reasons we conclude that, for indirect FISH, QD-conjugated streptavidin (at least in its current form) is an unsuitable material compared with equivalent Cy3 conjugates. For direct labelling, despite recruiting the services of leading proponents involved in QD conjugation (L. A. Bentolila, personal communication 2007), we were unsuccessful in generating a single successful FISH preparation by this means. It seems reasonable to suggest that, had we continued our attempts, we would eventually have met with a degree of success; however, given the intermittent success of the simpler indirect approach, we are not confident that the experiments would have been reliable. In addition, we have gone to the lengths of canvassing like-minded groups who would benefit from the use of QDs and organized symposia to share knowledge and experience. Without exception, the message we have received from our colleagues is of a similar experience to our own. In addition, recent studies (Bruchez 2007) also hint at the unreproducible nature of QDs for FISH and stress the need for tailored protocols established by empirical means. If this were achieved, then the reliability might well improve and the benefits of QDs observed in this and other studies (e.g. increased brightness, resistance to photobleaching) might be properly realized.

It is of course appropriate to speculate why QDs lack reproducibility in FISH applications. One possible explanation is their size. QDs vary in size (this is the basis of the fluorescent colour that they emit) from 2 to 10 nm. A Cy3 molecule on the other hand is <2 nm in size (Bailey et al. 2004). This may explain in part why our successful FISH experiments gave the impression of larger fluorescent particles and why there was a greater degree of background for most experiments. It might also explain the fluorescent ‘sheath’ effect seen on some metaphases (Fig. 3) and why certain preparations were successful at interphase but not at metaphase (Fig. 4). That is, steric hindrance may have led to signals being brighter in areas where the chromatin is less compact (e.g. at the edge of the chromosomes and/or in the interphase nucleus). If this were the case, we might have expected to see an improvement when we reduced the ratio of labelled to unlabelled dUTPs and/or when we made use of a ‘longer-arm’ biotin dUTP; however, we did not. Again a general background of intermittent success may have masked any appreciable difference seen in any given

experiment. The steric hindrance problem was reported also by Muller et al. (2006) in their attempts to use streptavidin-conjugated QDs to target plant chromosomes.

It is not entirely clear how streptavidin is bound to the polymer site of the QD; the number of free streptavidin sites per QD varies from 10 to 15 and they are prone to de-conjugation for reasons not completely understood (L. A. Bentolila, personal communication 2007). We are also aware that QD streptavidin conjugates can be prone to degradation (a batch-specific attribute) and this can correlate with even subtle changes in temperature during storage. Additionally, we are given to understand that QDs are prone to adhere to tubes sides and tips (P. Chan, personal communication 2005). Our attempts to reduce this problem using siliconized tubes and regular sonication met with a degree of success (confirming this theory in part), but did not completely eliminate our technical problems.

A further complicating factor was that the emission spectrum of the QDs used appeared to be not as narrow as the manufacturers claimed, in that we observed 'bleed-through' from red to green channels and vice versa, despite using narrow band-pass filters. Anecdotal evidence suggests that this phenomenon is not uncommon (L. A. Bentolila, personal communication 2007) and could vary from batch to batch. As we understand it controlling the size of the core during synthesis (which will determine the colour that the QD will emit) is an imperfect process and can lead to QDs being smaller or larger than expected. Moreover, abnormalities in QD shape (failure of quality control) could result in the same effect (L. A. Bentolila, personal communication 2007). Such a phenomenon can potentially lead to a mixed population of QDs in any given batch. These findings are consistent with the work of Bawendi and colleagues who have tried to address monodispersity of QD preparations (Murray et al. 2000). Supplementary Fig. S1 illustrates this phenomenon in that the different colours seen represent individual QDs that emit at longer wavelengths (towards the red—large QDs) or shorter wavelengths (towards the blue—small QDs). All these technical features that were attributed to the chemical synthesis of the QDs possibly require more experimental attention in order to improve QD synthesis.

Another observed QD feature was 'blinking', which is not seen in conventional FISH (as shown

in Supplementary Movie S2). Blinking is a phenomenon in which the QD alternates between an emitting (on) and non-emitting (off) state (Michler et al. 2000; Pinaud et al. 2006). This behaviour has been interpreted according to an Auger ionization model (Efros and Rosen 1997). Blinking affects single-molecule detection applications by saturation of the signal; however, one study suggests that this behaviour of the QD can be suppressed by passivating the QD surface with thiol groups (Hohng and Ha 2004). Photobrightening, wherein QD fluorescence intensity increases in the first stage of illumination and then stabilizes, can impose limitations on quantitative studies (Gerion et al. 2001). Both of these properties are associated with mobile charges on the surface of the QDs (Fu et al. 2005). It is also noteworthy that, although preparations often displayed blinking, they could go to an irreversible photodarkened state without easy explanation.

One possible explanation for the success of the groups that have published in this area (Xiao and Barker 2004a; Bentolila and Weiss 2006; Ma et al. 2008) is that they possessed the facility to synthesize and batch-test their own streptavidin QD conjugates (something that we, in common with most groups, do not currently have). In other words, they did not use commercially available streptavidin QDs. Ma (Ma et al. 2008) specifies that the QDs used were smaller than commercial ones, and that could help avoid steric hindrance and confer hybridization ability. Several authors (Xiao and Barker 2004a; Bentolila and Weiss 2006; Ma et al. 2008) used oligonucleotides to generate QD-DNA conjugates and highlight that, during the time of annealing of the QD-DNA probe to the target, steric hindrance has little effect but it may limit the QD's access to the target at the time of detection (Ma et al. 2008). This could also explain our negative results during direct FISH. A further complication of their application in biological environments is that QDs behave not as molecules but as nanocolloids (Resch-Genger et al. 2008).

Taking all of the above into consideration, the future of QD-FISH requires further research and interaction within the interested groups. Advances in nanomaterial synthesis (regarding uniformity and size control) and solubility will assist conjugation to biomolecules. Yao et al. (2006) described a new generation of nanocrystals called 'FloDots'. These are dye-doped silica nanoparticles that possess all QD

optical properties but, owing to the silica matrix that encompassed the dots, it is easier to make them water soluble and, according to the authors, the silica surface could be modified to contain functional groups for bioconjugation. In addition, a study by Choi et al. (2007) introduces a novel class of nanocrystals, 'C-dots', that could be 2–3 times brighter than QDs, less toxic and an ideal material for *in vivo* applications and cancer studies. Time will tell whether these or novel nanocrystals will be used robustly in FISH applications.

Nanotechnology has the potential to revolutionize the use of FISH in a wide range of molecular cytogenetic applications including gene mapping, clinical diagnostics, comparative genomics and microarray. The ability to multiplex much more effectively with a single excitation wavelength with bright, narrowly emitting fluorochromes that do not fade is highly desirable. QD-FISH will, in time, probably be seen as a significant stepping-stone towards this goal. Nanotechnology quite possibly holds the key to future of molecular cytogenetics. That future however, is not yet with us.

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