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Method for multiplex cellular detection of mRNAs using quantum dot fluorescent *in situ* hybridization

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ABSTRACT

The photostability and narrow emission spectra of non-organic quantum dot fluorophores (QDs) make them desirable candidates for fluorescent *in situ* hybridization (FISH) to study the expression of specific mRNA transcripts. We developed a novel method for direct QD labeling of modified oligonucleotide probes through streptavidin and biotin interactions, as well as protocols for their use in multiple-label FISH. We validated this technique in mouse brainstem sections. The subcellular localization of the vesicular monoamine transporter (Vmat2) mRNA corresponds when using probes labeled with two different QDs in the same hybridization. We developed protocols for combined direct QD FISH and QD immunohistochemical labeling within the same neurons as well as for simultaneous study of the subcellular distribution of multiple mRNA targets. We demonstrated increased sensitivity of FISH using QDs in comparison with organic fluorophores. These techniques gave excellent histological results both for multiplex FISH and combined FISH and immunohistochemistry. This approach can facilitate the ultrasensitive simultaneous study of multiple mRNA and protein markers in tissue culture and histological section.

INTRODUCTION

Radioactive and non-radioactive approaches for studying the localization of specific mRNA targets have a central role in biological research (1,2). Fluorescent *in situ* hybridization (FISH) techniques have been used to investigate the cellular and subcellular distribution of a variety of specific mRNAs in both tissue culture and in intact histological sections (3). Both synthetic RNA and DNA probes can be utilized and various direct and indirect labeling techniques have been developed (4). Because oligonucleotide probes are directly synthesized, they are in general advantageous in designing probes to distinguish splice variants or closely related sequences. In addition, oligonucleotide probe design and synthesis are scalable, potentially allowing transcriptome-level assays to be established for a large number of mRNAs for localization studies in anatomical sections. The limited signal intensity of end labeled oligonucleotide FISH probes has been addressed by the development of approaches that allow the post-synthesis addition of multiple fluorosphores to each probe via incorporate of aminoallyl modified nucleotides in the probe, a technique that can provide single mRNA molecule target sensitivity in cultured cells (5–7). Recently, this method has been modified for the study of the cellular and subcellular localization of multiple mRNAs in mouse brain tissue (8).

The organic fluorophores typically utilized for FISH have relatively broad emission spectra and show significant photobleaching, attributes that limit their use in signal quantification. The recent development of quantum dot (QDs) modified for use in biology provides an important new tool for signal detection in FISH (9,10). QD fluorophores have several potential advantages for FISH (11). Their narrow emission spectra facilitate the studying of multiple probes simultaneously (10,12,13). The use of QDs in multiple-labeling immunohistochemistry has recently been described (13,14). In addition, QDs are bright fluorophores that are essentially devoid of photobleaching, making them ideal for imaging low level mRNA signals. Photobleach-resistance is advantageous in studies of mRNA expression in tissue section due to the autofluorescence caused by tissue components. Unlike any signal from QDs, autofluorescence undergoes photobleaching with UV illumination (15). Therefore the specific QD signal could be easily distinguished from autofluorescence by light irradiation of the specimens.

Utilizing oligonucleotide probes for FISH is advantageous due to the flexibility in sequence design they provide. Water soluble hydroxylated QDs have been developed for labeling oligonucleotide probes for application in FISH assays in human sperm cells (16). Recently, QD-labeled RNA has also been applied in microarray assays for miRNA expression (17). The successful use of streptavidin-conjugated QD for detection of human metaphase chromosomes with biotinylated

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oligonucleotide has recently been described (18). The suitability of QDs for multiple target detection studies cannot be real ized by previous FISH protocols which rely on hybridization of a biotinylated probe and subsequent detection using streptavidin-conjugated QDs.

QDs have not been previously incorporated directly into oligonucleotide probes designed for FISH targeting specific mRNAs. We report the development of a novel multiplex FISH method using oligonucleotide directly labeled with inorganic streptavidin-conjugated QDs. Commercially available streptavidin-conjugated QDs can be used to label biotin-conjugated oligonucleotide probes for the detection of mRNA in tissue sections. Direct labeling of the biotinylated oligonucleotide to streptavidin-conjugated QDs is difficult because of the multiple sites of streptavidin on the QD core which therefore interfere with the hybridization efficiency of the oligonucleotide probe to the mRNA target. To overcome this limitation, we developed a novel strategy in QD oligonucleotide probe design and FISH protocol. This method allows multiplex detection of mRNA targets and provides high resolution histological results in tissue sections.

MATERIALS AND METHODS

Probe design and preparation

The QD-labeled oligonucleotide probe preparation and purification procedure is illustrated in Figure 1. Oligonucleotide probes specific for mouse vesicular monoamine transporter (Vmat2) mRNA and for ε-*sarcoglycan* (SGCE) mRNA containing 54 base carbon spacers at the 3′ end of the oligonucleotide and biotinylated tails were synthesized commercially (IDT DNA, Coralville, IA). The *Vmat2* sequence was 5′-GGAACTACGACGGTGAGCAGCATGTTGTCTAGCAGC-AGCGCGAGGAACAC-3′ and the SGCE sequence was 5′-TCCAACACATGAACAAAGAGGACACCTGCAGATG-GGTACACGTTCCGGTC-3′). Streptavidin-conjugated QDs of 525, 565, 585 and 605 nm were used for different directing labeling reactions (Quantum Dot Corp., Hayward, CA). The oligonucleotide probes were denatured for 10 min at 70°C. The conjugated QDs (100 nM) were sonicated in a Branson (Branson Ultrasonics Corp., Danbury, CT) for 10 min in a microfuge tube. The QDs were then incubated with 2 μM of biocytin, a water soluble biotin derivative (Sigma, St Louis, MO) for 20 min at 30°C in 50 mM borate buffer. Oligonucleotide probe (100 nM) was added to the streptavidin-conjugated QD and biocytin mixture and incubated for another 80 min at 37°C. The labeled probe was purified using a Superdex 200 column and the incorporation of QDs with the oligonucleotide probe was confirmed by agarose gel electrophoresis.

Animals and tissue preparation

An adult 129/Sv mice of 12–15 week with corpus callosum were maintained singly in cages in a 12 h light/dark cycle with *ad libitum* access to food and water. All procedures used in the study were conducted in accordance with the National Institutes of Health (NIH) guidelines for animal research and approved by the Center for Laboratory Animal Sciences at Mount Sinai School of Medicine. The animals were deeply anesthetized with pentobarbital (10 mg/kg i.p.) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS) (1× PBS, pH 7.4), followed by 4% paraformaldehyde (PFA) in 1× PBS. Brains were removed and post-fixed in 4% PFA for 6 h at 4°C. Brains were transferred to 10 and 20% sucrose in 1× PBS for 2 h each and then to 30% sucrose in PBS at 4°C for an additional 20 h. Twenty five microm coronal sections of mouse brain were prepared on a sliding microtome equipped with a freezing stage. The sections were stored in a 24-well tissue culture plate in 1× PBS at 4°C for immediate use or at −20°C in 30% sucrose solution for storage.

FISH protocol

All procedures for FISH were carried out on free-floating brain sections in 24-well tissue-culture plates. Sections were selected for FISH and adjacent sections were mounted on glass slide for NISSL staining for anatomical confirmation (8). Sections were treated with Proteinase K (0.5 μg/ml) at 42°C for 20 min. Before hybridization, the labeled QD oligonucleotide probe was heated to 70°C in the hybridization buffer for 15 min. Hybridization buffer containing 4× SSC, 50% formamide, 100 ng/μl purified BSA, 1× Denhardt's solution, 0.3% Triton X-100, 0.5 mM ribonucleoside vanadyl complexes and 1 ng/μl unlabeled scrambled probes (8) was pre-warmed to 42°C. Each QD-labeled (1 nM) of oligonucleotide probe was added to each well. Hybridization was carried out for 15 h at 42°C in a humidified incubation oven. After hybridization, the sections were washed three times for 30 min each in 1× SSC and 0.3% Triton X-100 at 60°C followed by 1× SSC and 0.3% Triton X-100 at room temperature for 1 h with gentle agitation. The sections were rinsed once with 0.1× SSC for 5 min and washed with PBSM (PBS with 50 mM $MgCl_2$) for 10 min. Nuclear counterstaining was carried out using 4′,6-diamidino-2-phenyindole (DAPI; 0.1 μg/ml) in PBSM and the sections were then rinsed once in PBSM for 5 min before mounting on glass slide. The mounted sections were dried in a 30°C oven and then coverslipped using mounting medium consisting of 90% glycerol and 1% *p*-phenylenediamine dihydrochloride in PBS and the edges sealed with nail polish.

Combined FISH and immunohistochemistry

FISH was performed as described above with direct processing for IHC after the PBS wash bfore DAPI staining. Sections were post-fixed with 2% PFA and rinsed with PBS. Immunohistochemistry using QD on mouse brain tissue was performed as described previously (8). Primary antibody for tyrosine hydroxylase (TH) (Sigma) was prepared at 1:1000 dilution and incubated for 2 h at room temperature with gentle agitation. Sections were washed five times for 10 min in PBS, followed by incubation with 1:500 dilution of biotin-conjugated IgG secondary antibody in PBS containing 5% BSA and 0.3% Triton X-100 at room temperature for 1 h. The sections were rinsed five times in PBS for 10 min. The CdSe/ZnS streptavidin-conjugated fluorophore QD 605 was diluted to 10 nM in PBS and incubated for 50 min, followed by two rinses in PBS for 10 min. Sections were counterstained for nuclei with DAPI, mounted on glass slides, dried and coverslipped as described for FISH above.

Multi-spectral FISH

Hybridization was performed with 1 ng/μl Alexa fluorophore labeled probe of dopamine D2 receptor (probe 1: **T**AAAGATGAGGAGGG**T**GAGCAGCA**T**GGCATAGTAGTTGT AGTGGGGCC**T**G; probe 2: CCCA**T**GGGATTC**T**ATAACAG**T**GGAGAACATAGC AA**T**ACCCACTGCTA**T**GG), TH (probe 1: **T**GAGTGCATAGG**T**GAGGAGGCA**T**GACGGATGTAC**T**GTGTGCAC**T**GAAACA; probe 2: **T**CAAAGGG**T**CCAGCCACACAC**T**GGGAAAGCC**T**CTGGGCCAGGAAAGG**T**T), boldface letters represent regions containing amino-modified nucleotides which were then labeled with succinimidyl esters Alexa fluorophores. The Alexa fluorophore probe labeling and purification were performed as described previously (8). The hybridization was performed at the same condition as described above.

Microscopy

Fluorescent imaging was performed with a Zeiss LSM510-META inverted confocal laser scanning microscope equipped with a PC containing the LSM software for control of all system components. Imaging was achieved using a ×63/1.3 NA or ×100/1.3 NA oil immersion objective. For imaging the QDs and DAPI signals, the blue diode laser 405 nm was used for simultaneous visualization of DAPI, QD 525 and QD 585 with bandpass emission filters of 420–480, 505–530 and 560–615, respectively. For the detection of DAPI, QD 525 and QD 605, the band past emission filters of 420–480, 505–530 and 560–615 were used, respectively. For imaging the QD, DAPI and Alexa organic fluorophore signals, the blue diode 405 laser was used for the excitation of QD 565, QD 605 and DAPI, the HeNe laser 543 was used for excitation of Alexa 568 and an argon laser 488 was used for the excitation of Alexa 488. Lambda stacks were acquired to determine the reference spectra. The five fluorescent signals were then separated using the Linear Unmixing function in the LSM 510 META confocal microscope. Epifluorescent microscopy was performed on an Olympus BX60 microscope equipped with a BX-FLA Reflected Light Fluorescence Attachment and a CCD-based image analysis system. Each image field was captured as a digital image using the SPOT Advanced system (Diagnostic Instruments, Sterling Heights, MI).

Data analysis

Quantitative analysis of fluorescent signal on image captured from LSM confocal microscope was performed by using the NIH Image J program. The fluorescent signal from FISH labeled cell was measured at cell level from images taken at ×100 magnification.

RESULTS

Probe design and purification

The large size of conjugated QDs appear to interfere with hybridization efficiency when used in direct oligonucleotide-labeled FISH (11). In order to overcome this limitation, we developed a labeling strategy using a 54 carbon spacer between the biotinylation site and the oligonucleotide probe (Figure 1). Because the commercially available streptavidin-conjugated QDs contain multiple streptavidin sites, the

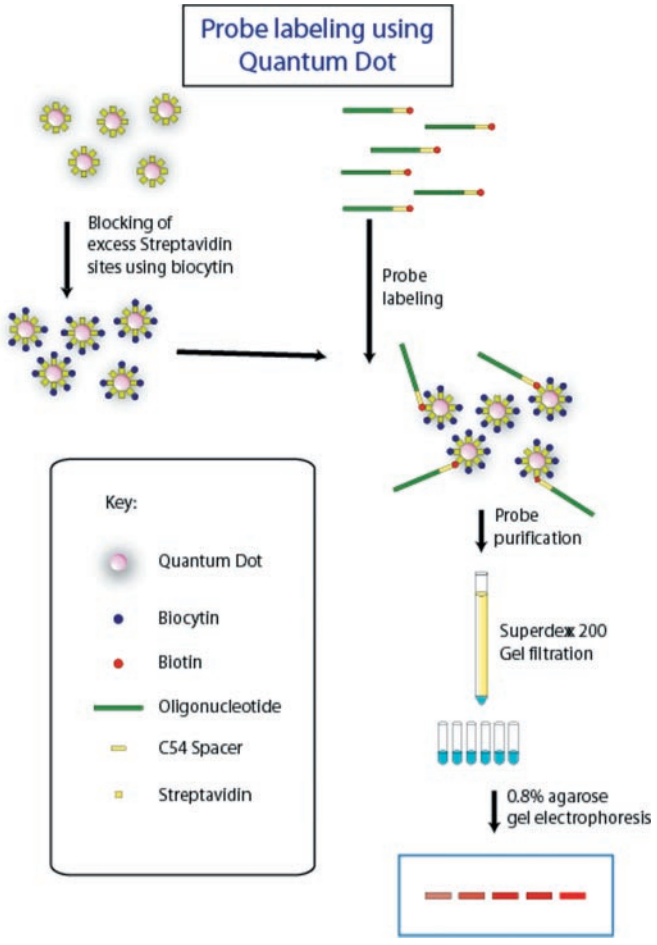

Figure 1. (**A**) Schematic representation of quantum dot oligonucleotide probe labeling strategy for FISH. Excess streptavidin sites on the QDs were first blocked by biocytin before adding to the biotinylated oligonucleotide for labeling. The QD labeled oligonucleotide probes were purified using a Superdex 200 column.

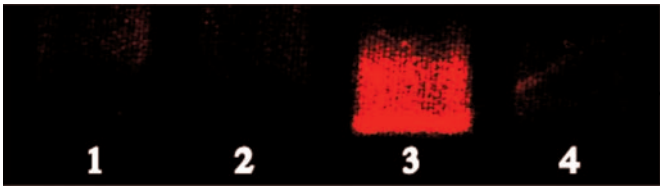

Figure 2. Agarose gel electrophoretic analysis of biotinylated oliogonucleotide probes with streptavidin-conjugated QDs 585. The individual samples represent fractions eluted from a Superdex 200 column. The Superdex 200 purified oligonucleotide was used directly for FISH. High-molecular weight conjugates eluted in later fractions (data not shown).

streptavidin-conjugated QDs were first incubated with a titrated concentration of biocytin in order to reduce the number of streptavidin sites available for labeling which would otherwise lead to massive oligonucleotide crosslinking. The QDs were then incubated with oligonucleotides containing the 54 carbon spacer and 3′ biotin tail. The mixture of oligonucleotide and QDs were purified using a Superdex 200 column, the fractions were collected and analyzed on a 0.8% agarose gel before visualized by UV lamp (Figure 2). The fraction containing the smallest molecular weight band

on the gel, which are enriched in mono-oligonucleotide-complexed QDs, were used for FISH.

Single QD FISH in mouse brain section

Results of single label FISH in mouse brain sections with QD-labeled oligonucleotides are shown in Figure 3. Oligonucleotides targeted for *Vmat2* were labeled with either QD 525 or QD 585. Signal specificity was confirmed by eliminating signal when QD-labeled probe was hybridized in the presence of excess-unlabeled oligonucleotide (data not shown). In Figure 3A, QD 525 labeled neurons in the dopaminergic neurons in substantia nigra (SN) were visualized with a confocal microscope using a blue laser 405 nm for excitation and the signal was detected using the band pass filter at the 505–530 nm (Figure 3A) and the DAPI signal for the nuclei was detected at 420 nm (Figure 3B). The labeling of the *Vmat2* mRNA signal was restricted to the midbrain neurons of the SN in the tissue section. Figure 3C shows the overlay of QD 525 and DAPI. Note the bright *Vmat2* mRNA QD 525 fluorescent signal in cytoplasm and the absence of QD 525 labeling in the nuclei. In Figure 3D, the *Vmat2* oligonucleotide probe was labeled with QD 585 and the mRNA signal was detected at 560–615 nm. The *Vmat2* mRNA signal in dopaminergic neurons in SN was intense and the cytoplasmic distribution of the mRNA could be detected. Tissue background was low and the *Vmat2* probe gave a specific and sensitive FISH signal.

We compared the sensitivity of QD FISH to that of a highly sensitive multiple-label organic fluorophore FISH protocol that we have utilized previously (8). This organic fluorophore protocol uses multiple probes designed against the same mRNA target. Using a combination of three probes for mouse ε-sarcoglycan mRNA, which are each labeled with five Alexa 488 fluorophores, we have described previously the excellent histological results obtained with photomicrographs obtained at ×20 magnification (8). However, as shown in Figure 4A, at ×4 magnification this highly sensitive organic fluorophore FISH protocol cannot detect neurons in the mouse midbrain region that are known to express this mRNA target (8). We compared this result to that obtained using QD FISH with a probe of identical sequence to that of one of the organic fluorophore FISH probe set. This probe was attached to a single QD fluorophore and images were obtained at the same magnification and exposure as that for the organic multiple-label FISH. In contrast to the results obtained with the multiple-label organic fluorophore FISH assay, which to our knowledge is the most sensitive FISH assay described previously (5,6), the QD FISH assay clearly detects labeled neurons in the appropriate regions of the midbrain section (Figure 4B). These results indicate the increased sensitivity provided by the use of QDs for FISH.

Multiple labeled FISH

In this study, we have found that the same mRNA signal detected with the QD probes could be very different in fluorescent intensity when different lasers were used for QD excitation. We tested the excitation of QD 525/QD 585 using a diode 405 nm blue laser, an argon (488 and 514 nm) and a red HeNe (633 nm) lasers. For the same cell labeled with QD 525 *Vmat2* probe, the fluorescent signal excited by the 405 nm line is ∼6-fold and 4-fold higher than those excited by 633 and 514 nm lines, respectively, using the same laser power settings (data not shown). For the QD 585 labeled *Vmat2* probe, excitation using the blue diode 405 nm laser produced 4-fold higher fluorescence intensity than the signal obtained by the argon 514 laser. Multiple QD FISH was performed by labeling the *Vmat2* oligonucleotide probe with two different QDs and performing hybridization simultaneously with both probes.

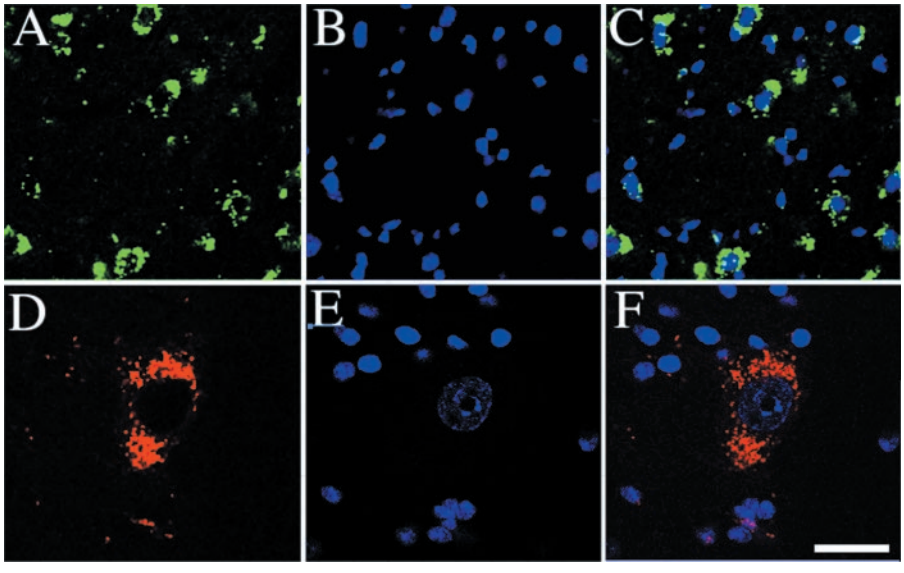

Figure 3. Fluorescent labeling of cellular mRNA in mouse brain sections using QDs. (**A**) Dopaminergic cells in SN labeled with *Vmat2* oligonucleotide containing QD 525. (**B**) DAPI staining of nuclei. (**C**) Overlay of the QD 525 and DAPI signals. (**D**) High magnification image of cell in SN labeled with *Vmat2* QD 585 oligonucleotide probe. Note the specific cytoplasmic labeling of the *Vmat2* mRNA and the low tissue background. The images were obtained from 25 μm tissue sections and captured using a ×100/1.3 NA objective with ×4 digital zoom. Simultaneous detection of DAPI, QD 525 and QD 585 signals were excited by a diode 405 laser on a Zeiss Meta LSM. Scale bar = 25 μm for (A–C) and 10 μm for (**D–F**).

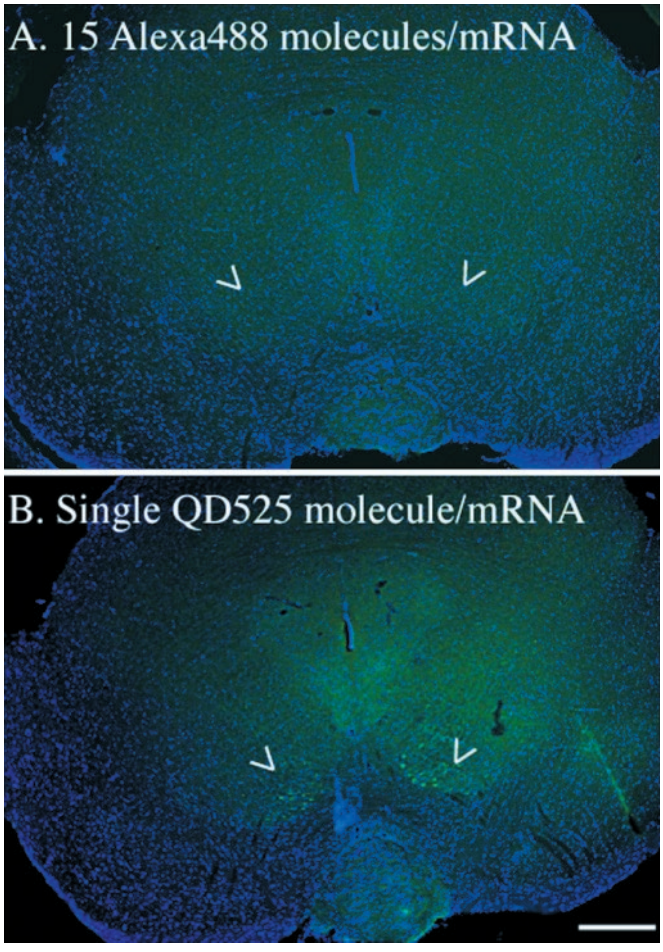

Figure 4. Comparison of organic fluorophore and QD FISH sensitivity. (**A**) Mouse midbrain section hybridized with three probes for ε-sarcoglycan mRNA (SGCE) each labeled with five Alexa 488 fluorophores. In our hands, this protocol gives excellent histological results for ×20 images of this mouse brain region, as described previously (8). However, at ×4 magnification, neurons that are known to express this mRNA within the mouse red nucleus are not able to be detected (arrow heads). Alexa signal is green and DAPI nuclear counterstain is blue. (**B**) The same region studied with one of the three ε-sarcoglycan mRNA probe sequences used in (A), labeled with a single QD 525 fluorophore. Neurons in the red nucleus that express this mRNA are clearly visible (arrow heads). QD 525 signal is green and DAPI signal is blue. Scale bar = 500 μm.

The QD525, QD 585 and DAPI signals were detected simultaneously using a single 405 nm laser. The *Vmat2* mRNA was detected with both QD 525 and 585 nm (Figure 5A and B, respectively). Note the colocalization of the *Vmat2* QD 525 and QD 585 signals in the mouse midbrain. The QDs signals were only detected in the cytoplasm and were absent in the nuclei. Control experiments were performed using unlabeled *Vmat2* oligonucleotide probes in the prehybridization and hybridization and the results demonstrated the elimination of any specific fluorescent signal (data not shown). These data indicate that this labeling and hybridization QD FISH protocol generates specific and sensitive mRNA detection with low tissue background.

We also investigated the use of multiple QD and organic fluorophores for multi-spectral simultaneous detection of up to four different mRNA targets within the same cells. We

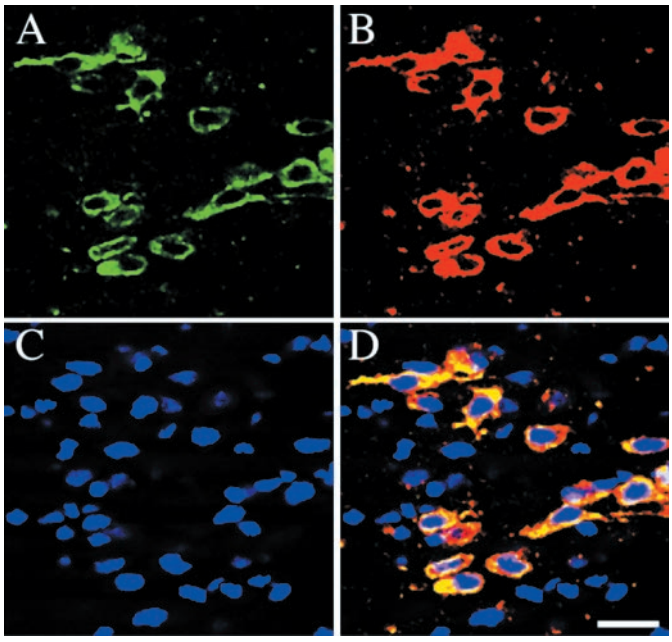

Figure 5. Double labeling FISH using QD 525 and QD 585 oligonucleotide probes. Neurons in SN were labeled with two different QD oligonucleotide probes. *Vmat2* mRNA was detected using both QD 525 (**A**) and QD 585 (**B**) probes. The fluorescent signals for DAPI (**C**) and QDs were detected simultaneously using a diode 405 blue laser of the Zeiss Meta 510 LSM. The FISH signal was detected only in cytoplasm and low background signal. Note the complete overlap of QD 525 and QD 585 signal (**D**). Scale bar = 20 μm.

were able to detect simultaneous expression of dopamine D2 receptor mRNA, ε-sarcoglycan mRNA, TH mRNA and *Vmat2* mRNA using two different QD fluorophores and two different organic fluorophores within a single mouse midbrain neuron (Figure 6A–E). At high magnification, a triple-labeling study for ε-sarcoglycan mRNA, TH mRNA and *Vmat2* mRNAs using two QD probes and one organic probe revealed a unique pattern of subcellular distribution for each mRNA target (Figure 6F).

QD FISH and QD immunohistochemistry

We also investigated whether it was possible to combine QD FISH and immunohistochemistry in order to study the cellular and subcellular comparative localization of mRNA and protein epitope targets. After tissue sections had been processed for QD FISH, the same sections were used for immunohistochemistry using a different streptavidin-conjugated QD. In order to evaluate the specificity of combined QD FISH and QD immunohistochemistry, we studied mouse midbrain sections which are known to contain dopaminergic neurons expressing both *Vmat2* and TH (19). The results of QD FISH for *Vmat2* mRNA and immunohistochemistry for TH are shown in Figure 7. *Vmat2* mRNA positive cells were labeled with oligonucleotide-labeled with QD 525 (Figure 7A). TH protein was detected in the same neurons using streptavidin-conjugated QD 605 (Figure 7B). Nuclei were labeled with DAPI (Figure 7C). A single blue laser was used to excite all the three fluorophores. The *Vmat2* mRNA detected in Figure 7A is restricted to the cytoplasm. The DAPI labeled

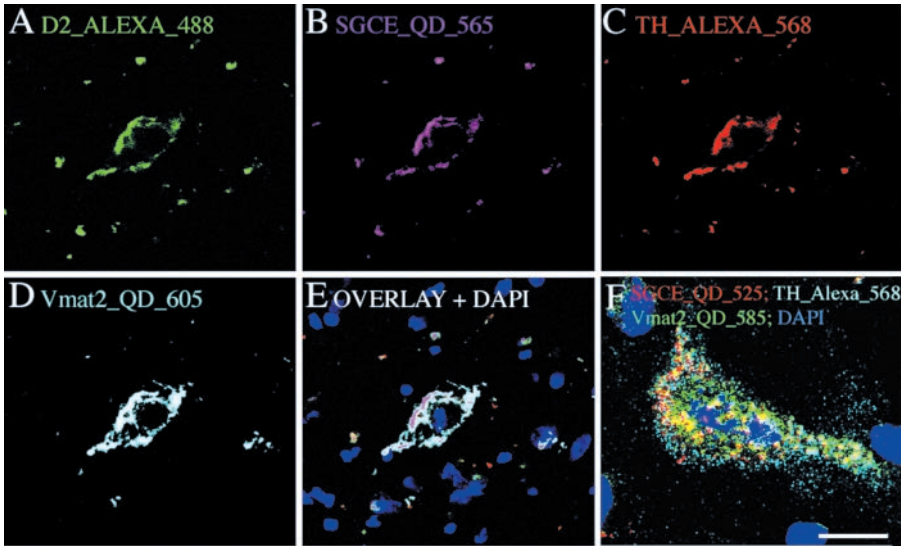

Figure 6. Multiple-label and multi-spectral FISH using QD and organic fluorophores. Probes were labeled with the fluorophores indicated and hybridization for multiple mRNA targets was performed. (**A**–**D**) Detection of four different mRNA targets separated by spectral imaging of a 0.2 μm optical section. The emission of each individual fluorophore was clearly separated. (**E**) Overlay of (A–D). (**F**) Overlay of higher magnification spectral imaging of a single neuron labeled for three different mRNAs. Note the differences in the subcellular pattern of each mRNA target. DAPI counterstaining of the same sections is blue. Scale bar: (A–E) = 20 μm; (F) = 10 μm.

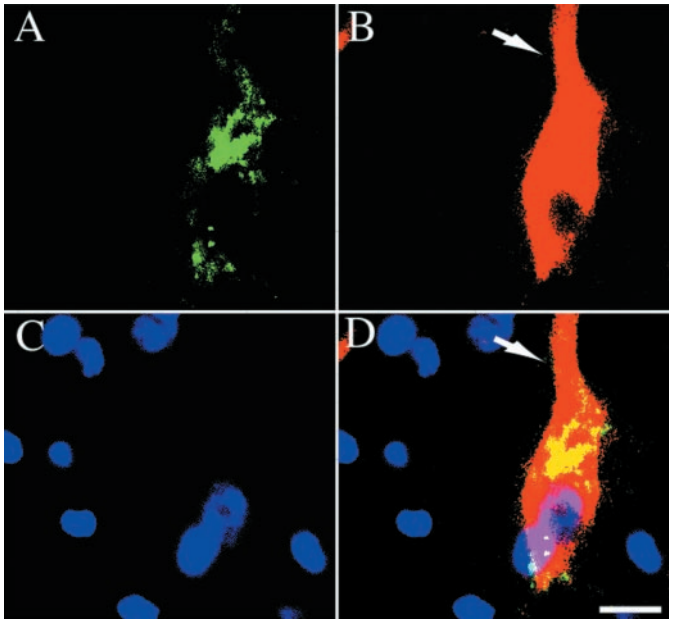

Figure 7. Simultaneous multiplex labeling of mRNA and protein in mouse brain using QD 525 and QD 605. *In situ* hybridization was performed and the same tissue sections were processed for immunohistochemistry using QD for the detection of protein epitopes. (**A**) *Vmat2* mRNA positive neurons in SN was labeled with QD 525. (**B**) The same cell was labeled with TH antibody and the signal was detected using streptadvidin conjugated QD 605. Note the labeling of both *Vmat2* mRNA and TH protein in the same cell. The *Vmat2* mRNA signal is restricted to the cytoplasm whereas the labeling of TH is extended to the whole cell body and processes (arrow). (**C**) DAPI labeling of nuclei. (**D**) Overlay shows the differing subcellular distributions of *Vmat2* mRNA and TH immunoreactivity. Scale bar = 15 μm.

only the nuclei and the TH signal was distributed throughout the cytoplasm and neurites. The combined labeling study showed co-expression of *Vmat2* mRNA and TH immunoreactivity in the same cell (Figure 7D).

Photostability analysis of QD FISH in brain section

We next evaluated the photostability of QD FISH signals. Neurons in the dorsal motor nucleus of the vagus of the mouse brainstem labeled with *Vmat2* QD 585 oligonucleotide are shown in Figure 8. The fluorescent signal in the digital confocal image obtained was quantified using the NIH Image J program (Figure 8). After 1 h photobleaching, an image of the same section was captured and the fluorescent signal quantified (Figure 8C). The signal intensity curve is shifted downwards slightly due to photobleaching of tissue autofluorescence, while the specific QD signal is completely photobleach resistant. These data indicate that the QD FISH protocol provides stable and sensitive labeling of specific mRNAs in brain sections, while preserving high histological quality.

DISCUSSION

Identification of critical morphological steps during development and cellular studies of complex tissues such as the brain rely on specific molecular markers from which potential biological and functional relationships can be established. Although microarray and quantitative real time PCR technologies have been developed for high-throughput analysis of thousands of transcripts simultaneously, both methods require extraction of RNA from tissues which limits their application in molecular anatomical analysis where histological preservation of sample is essential (20). The use of QDs for the study of multiple mRNA targets in tissue will facilitate the development of single cell genomics analysis of complex tissues.

The novel method described here demonstrates a simple FISH methodology for highly sensitive and specific detection of mRNA targets. QD FISH allows detection of mRNA by labeling oligonucleotides with different QDs at different emission wavelength. We have demonstrated that this method

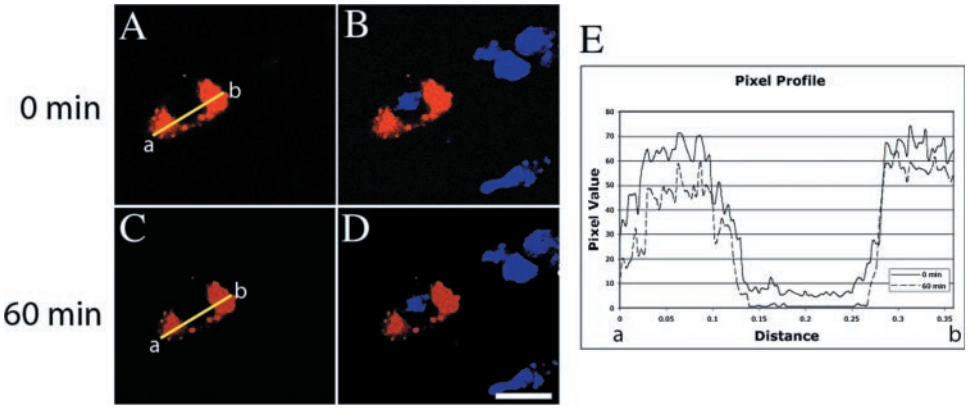

Figure 8. QD FISH probe is resistant to photobleaching. **(A, B)** A neuron in DMX was labeled with QD 585 and the image was captured using the 405 nm laser at time 0 min. (**C,D**) The cell was continuously irradiated by the blue laser for another 60 min and the image was captured. The settings for laser power, gain, offset, scanning time and pinhole were identical for both images and the fluorescence emission was recorded through a ×100 NA 1.3 oil objective. (**E**) Fluorescence intensity across the same cell coordinate was measured using NIH Image J program. Scale bar: (A–D) = 15 μm.

can be combined with immunohistochemistry for detection of protein labeling for histological analysis. We have successfully applied this method to compare the cellular distribution patterns of the *Vmat2* mRNA and immunoreactivity of TH in dopaminergic neurons. This study revealed differential distribution of *Vmat2* mRNA and TH immunoreactivity in neurons, a subtle result which benefited from the high sensitivity of the QD fluorophores utilized. Therefore, the ability to use QD-labeled oligonucleotide probes to detect mRNA signal and immunoreactivity simultaneously in brain tissue provides a new level of sensitivity and specificity for studies at the interface of genomics and neuroanatomy.

Assay sensitivity is essential for the detection of low abundance mRNA molecules, such as those present in dendrites (21). Because QDs are photostable, they allow very long exposures and provide high levels of target mRNA sensitivity. QDs also have a broad excitation range, providing flexibility in the wavelength used for excitation. In addition, the development of QDs in the near infrared range, a frequency which penetrates tissue, provides fluorophores well-suited for the detection of molecules in thick sections. The high signal-to-noise ratio achieved in this QD FISH protocol has improved substantially the resolution of images therefore making it possible to observe the details of mRNA expression pattern at the subcellular level (Figure 6). Moreover, the high sensitivity and signal stability of this QD protocol will facilitate the use of automated image analysis systems for high-throughput applications (22,23).

The direct QD FISH method has several advantages in comparison with other approaches for FISH. First, fluorescence coming from directly QD oligonucleotide-labeled mRNA is likely to provide more accurate quantitation of relative levels of mRNA expression. An indirect detection of mRNA signal via a biotin–streptavidin interaction between the hybridized probes and streptavidin-conjugated QD can result in aggregation of the QD, altering signal intensity. Second, QD probe labeling and purification are relatively simple. After purification, the probes can be stored for months or longer without affecting the hybridization or histological results. Third, the FISH protocol is simple due to the direct labeling of QD to the oligonucleotide probes and the absence of amplification steps. Finally, the high photostability of QD probes facilitates transcript analysis and quantitation due to the signal stability not obtainable with conventional organic fluorophores.

All multiplex fluorescence techniques rely on the ability of the detection apparatus to separate the light emitted by different fluorescent dyes which label the specimen. Unfortunately, the emission spectra of most fluorophores are broad so that when multiple fluorophores are used together, their emission spectra are difficult to deconvolute. Innovations in microscopy hardware and software partly address the problem of spectral overlap. Linear unmixing has been implemented in the latest generation of confocal microscopes from Zeiss (LSM510-META), allowing the separation of multiple fluorophores. However, when dealing with brain tissue, endogenous autofluorescence contributes to a shift of the spectrum peak from different fluorophores and affect the reliability of linear unmixing when using organic fluorophores. The spectroscopic properties of QDs are fundamentally different from those of organic fluorophores. QDs have broad excitation profiles and narrow emission peaks and can be tuned to emit a range of wavelength by changing the size of the nanoparticles and their compositions. We also find it possible to combine QD and organic fluorophore labeling in order to increase the number of probes that can be distinguished using organic fluorophores alone as well as increasing the sensitivity for the targets detected with the QD fluorophores (Figure 6). The properties of QDs increases the number of probes that can be studied simultaneously using bandpass filters or linear unmixing algorithms.

The unique stability and spectral properties of QDs have led to their rapid adoption for a variety of molecular imaging applications. The development of a reliable QD FISH protocol provides a tool that facilitates the high-throughput multiplex study of gene expression at cellular and subcellular resolution in histological section.

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