Histol Histopathol (2007) 22: 617-622

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Autometallographic tracing of quantum dots

M. Stoltenberg, A. Larsen, P. Doering, E. Sadauskas, L.J. Locht and G. Danscher

Neurobiology, Institute of Anatomy, University of Aarhus, Aarhus C, Denmark

Summary. A short clarifying view of how semiconductor quantum dots (QDs) can be made visible in tissue sections by autometallographic (AMG) silver enhancement and how the introduction of AMG enhanceable gold nanoparticles into isolated cells can be used to follow the fate of these marked cells in organisms and cell cultures. As the AMG approach for visualizing quantum dots is extremely sensitive, QDs less than one nanometer can be made visible at both LM and EM levels.

Key words: Autometallography (AMG), Gold nanocrystals, Quantum dots, Semiconductor, Cd-Se, Cd-S, Zn-S, Pb-S

Introduction

In modern biological analyses, various kinds of organic dyes are used to tag cells and subcellular structures (Josephson et al., 1999; Dahan et al., 2003; Gao et al., 2004). However, sometimes traditional dyes are simply unable to meet the necessary standards, and quantum dots (QDs) are now taking over the role of the fluorescence dyes because they seem to be superior to traditional organic dyes in several ways, the most obvious being their brightness and their photostability (Bruchez et al., 1998). A quantum dot (QD) is a nanocrystal just a few nanometers in diameter, and the QD approaches therefore hold eminent possibilities for future biological studies (Bruchez et al., 1998; Warburton et al., 2000; Banin and Millo, 2003; Michalet et al., 2005).

QD tagged antibodies have already been implemented in a lot of studies (Wu et al., 2003; Alivisatos, 2004; Dahan, 2005; Rieger et al., 2005). In embryology, the QDs may revolutionize the field, especially due to the possibilities for in vivo imaging of tagged molecules and cells (Dubertret et al., 2002; Gao et al., 2004; Rieger et al., 2005; Stroh et al., 2005). The option of tagging isolated cells, introducing them into an organism and following them and their progeny for generations is another promising perspective (Danscher, 2003).

There are, however, rather problematic issues to be handled before the use of QDs becomes really popular. One thing is that most QDs are extremely toxic (Hardman, 2006), another that most of the QDs are unstable in water and therefore need coating (Bruchez et al., 1998, Dubertret et al., 2002; Bentolila and Weiss, 2003; Fu et al., 2005; Medintz et al., 2005; Michalet et al., 2005).

Several articles have been published on the benefit of using QDs that have semiconductor qualities. An important property of these QDs is that they release light of a defined wavelength if radiated with light. This quantum-defined release of photons is repeatable and therefore has the potential of being an important tool in histochemistry (Empedocles and Bawendi, 1999; Chan et al., 2002; Wu et al., 2003; Alivisatos, 2004; Dahan, 2005; Rieger et al., 2005). Another important dimension of these QD probes might be that they can be AMG silver enhanced. At the moment the most used semiconductor QDs are the core-shell CdSe/ZnS (Medintz et al., 2005), as well as Cd-S, Zn-S, Cd-Te, Cd-S, Pb-S, and Pb-Se nanocrystals, and they are all commercially available. Silver enhancement of QDs has previously been predicted as feasible (Danscher et al., 1995; Bruchez et al., 1998), and recently Dahan et al. (2003) showed that QDs can be silver enhanced and traced in the electron microscope. Unfortunately the authors did not describe how they performed the enhancement.

Gold nanoparticles do not have the light emission properties. However, gold as well as the semiconductor QDs can be silver enhanced by autometallography (AMG).

The principles of AMG are that the QD containing tissue sections are exposed to an AMG developer that contains silver ions and reducing molecules. The silver ions adhere to the surface of the catalytic QD and become part of the crystal lattice; they are then reduced to silver atoms by taking up electrons released from the reducing molecules that likewise connect to the QD. The

Offprint requests to: Meredin Stoltenberg, MD, PhD, Department of Neurobiology, Institute of Anatomy, University of Aarhus, DK-8000 Aarhus C, Denmark. e-mail: ms@neuro.au.dk

result of this process is a genuine growth of the QD particles (for a review see Danscher and Stoltenberg, 2006). We believe that AMG is the most suitable way to silver enhance QDs, also because the technique is easy to set up and control.

Materials and methods

Animals

Twenty-four female mice of the Balb/c strain were used. The animals were kept in plastic cages in a room with a 12-hour light/dark cycle at 21-22°C and 50% humidity. They were fed Altromin No. 1324 (Spezialfutterwerke, Germany) ad libitum and had free access to tap water. The study was undertaken in accordance with the Danish and University of Aarhus guidelines for animal welfare.

The animals were divided in 6 groups of 4 animals. Group 1: Creation of zinc-sulphur quantum dots by transcardial perfusion with a sodium sulphide solution (according to the NeoTimm autometallographic technique). Group 2: *In vivo* creation of zinc-selenium quantum dots (according to the selenium autometallographic technique). Group 3: Injection of cadmium-selenium quantum dots (0.1 ml intraperitoneal injection of CdSe; 6.2 mg/ml ~ 41,05 nmol/ml). Group 4: Injection of zinc-sulphur /cadmium-sulphur quantum dots (0.1 ml intraperitoneal injection of ZnS/CdSe; 2.2 mg/ml ~ 11.50 nmol/ml). Group 5: Injection of lead-sulphur quantum dots (0.1 ml intraperitoneal injection of PbS; 2.5 mg/ml). Group 6: Blank controls, i.e. no injection of quantum dots. Groups 3, 4, 5 were sacrificed after 2 hours.

Buffy coat cells (obtained from Skejby Sygehus, Aarhus University Hospital, DK-8200 Aarhus N, Denmark) were diluted 1:4 in PBS. The cell suspension was then mixed 1:1 with the colloidal Gold-Tat solution and stored at room temperature for 1 hour, prior to fixation in an equal volume of 6% glutaraldehyde in 0.2 M phosphate buffer for 30 minutes.

The cell suspension was centrifuged at 3000 rpm for 3 minutes and resuspended in 0.1 M phosphate buffer (pH 7.4). This procedure was repeated 3 times. The pellets were mixed with the autometallographic developer (for details see e.g. Danscher, 1981; Danscher and Stoltenberg, 2005, 2006) and placed in a dark box for 60 minutes. After AMG the cells were centrifuged and resuspended in buffer 3 times in all. 0.5% osmium tetroxide (1 ml 0.2 M buffer + 0.5 ml 2% osmium tetroxide + 0.5 ml dist. water) was added for 30 minutes. Finally, the cells were centrifuged and resuspended in buffer 3 times. The tube with the cell pellets was transferred to a 50°C water bath, and a 1 ml 2% agar (Difco; microbiological reagent) solution (60°C) was added and quickly mixed with the cells. The agar-cell solution was centrifuged once before the cell pellets were placed in 3% glutaraldehyde until Epon embedding. Semithin (3 μm) sections were cut and placed on glass slides. After light microscopic analyses, selected sections were re-embedded in Epon. Ultrathin sections were made and counterstained with lead citrate (26.6 g lead nitrate (Merck; p.a.), 3.52 g tri-sodium citrate dihydrate (Merck; p.a.), 16 ml 1 N NaOH, (Bie & Berntsen, Søren Nymarksvej 10 DK-8270 Højbjerg), in 100 ml distilled water) and saturated uranyl acetate (15 gram of uranyl acetate dihydrate (Merck p.a.) in 200 ml distilled water stored overnight in an ultrasound bath) before examination in the electron microscope.

Macrophages: J774A.1 (ATCC number TIB-67) cells were maintained under standard culture conditions. In brief, they were grown in an RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1% non-essential amino acids. Cells were cultured in 200 ml plastic flasks (NuncTM, Denmark) and subcultivated at confluence twice a week. The medium was changed every 1 or 2 days.

A Qtracker kit (Quantum Dot Corporation) was used to prepare the QD containing medium. 12.5 μl Qtracker Reagent A and 12.5 μl Reagent B were mixed and incubated for 5 min at room temperature. 1 ml of the standard cell culture medium was added and the solution mixed for 30 sec at high speed.

Each pellet consisted of approximately 10 million cells. The pellets were made by harvesting the confluent monolayer of cells grown in a 200 ml growth flask (Nunc™ , Denmark). 5 ml fresh cell medium was added, and a cell scraper ("rubber policeman") was used to loosen the cells. The cells were centrifuged for 5 min at 600 rounds per min (rpm) in a Selecta centrifuge. The entire medium was removed and the cells were re-suspended in 1 ml QD containing medium for 1 hour. After exposure, the cells were re-centrifuged for 5 min at 600 rpm, the QD containing medium was removed and 5 ml of the new medium added in order to wash the cells. The washing procedure was repeated twice using 0.1 M PBS buffer. After the washing procedure the pellets were fixated by exposing them to a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in a 0.1 M PBS buffer for 20 min. The pellets were then embedded in agar (2%), dehydrated, embedded in Epon, sectioned, subjected to AMG development for 70 min, and processed for electron microscopy as described above.

Chemicals

A Qtracker Cell Labeling Kit was used (Quantum Dot Corporation, catalogue number 2502-1). According to the manufacturer's specification the Qtracker Cell Labeling Kit delivers fluorescent QDs into the cytoplasm of live cells using a customary targeting peptide. Once inside the cells, the Qtracker provides an intense, stable fluorescence that can be traced through several generations and is not transferred to adjacent cells in a population (www.qdots.com).

The following commercially available quantum dots were used: CdSe, CdSe/ZnS (shell ZnS, core CdSe), Pb-S (Evident Technologies, catalogue numbers ED-C10-TOL-0620; ED-C11-TOL-0620; ED-P20-TOL-0850).

A peptide containing the translocation sequence of the Tat peptide (Biopetide Co., LLC) was used. The peptide is referred to as Tat, and the amino acids correspond to residues 48-57 of the Tat protein. The sequence is Gly-Gly-Cys-*Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg*-Lys- (Josephson et al., 1999). 0.5 mg Tat was dissolved in 1 ml distilled water and mixed with 2 ml of a colloidal gold solution (vide infra).

Creation of ZnS and ZnSe quantum dots in experimental animals

ZnS quantum dots

A zinc ion specific version of Timm's original sulphide silver method (Timm, 1958), "the NeoTimm method" (Danscher, 1981), was used for in vitro binding of zinc ions as zinc sulphide QDs (for details see also Danscher and Stoltenberg, 2006). This involves: Transcardial perfusion with a prewash of 5 ml 0.1% sodium sulphide solution (the NeoTimm solution, NTS) followed by 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 minutes and eventually NTS for 7 minutes without interrupting the flow. The brains were removed and postfixed for one hour in the glutaraldehyde solution.

ZnSe quantum dots

The autometallographic selenium method $ZnSe^{AMG}$ (for details see Danscher, 1982; Danscher and Stoltenberg, 2005, 2006) was used for *in vivo* creation of ZnSe QDs in mice. The animals were anaesthetized with a ketaminol/narcoxyl solution and injected 20 mg per kg bodyweight sodium selenite (Na_2SeO_3) intraperitoneally. The sodium selenite was dissolved in distilled water in a concentration of 1 mg sodium selenite per 1 ml dissolved water. Two hours after the selenite exposure the animals were deeply anaesthetized and sacrificed by transcardial perfusion with 3% glutaraldehyde in a 0.1 M phosphate buffer for 10 minutes. The brains were removed and postfixed for one hour in the glutaraldehyde solution.

Gold nanoparticles

Solutions containing different sizes of colloidal gold particles are commercially available (Fitzgerald Industries Int., US), but can also be prepared in the laboratory. E.g. a solution of 6 nm gold nanocrystals can be made in the following way:

Solution A: 4 ml of 1% $HAuCl_4$ + 316 ml distilled water.

Solution B: 16 ml 1% Na_3-citrate + 2 ml 1% tannic acid + 2 ml 25 mM K_2CO_3 + 60 ml distilled water.

Solutions A and B are both heated to 60°C. Then under constant stirring solution B is added to A (and turns red), and the temperature is allowed to rise to 100°C. The solution is kept at 4°C in a container wrapped in tinfoil.

Fluorescence microscope

Tissue sections were examined under a conventional fluorescence microscope (Olympus BX51 microscope; excitation filter 470 to 490 nm; emission filter >510 nm).

Results

The studied commercial and *in vivo* created metal-sulphur and metal-selenium quantum dots were

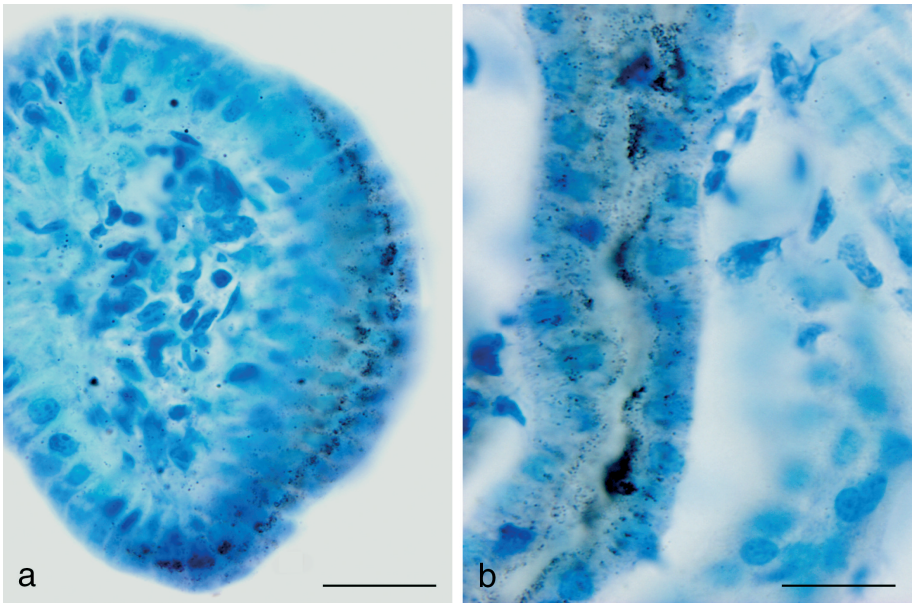

Fig. 1. Light micrograph of AMG silver enhanced CdSe QDs located in the epithelia lining the small intestine. The animal received a single intraperitoneal injection (ip) of CdSe and was allowed to survive for 2 hours. Bar: 20 μm. b) Light micrograph of AMG silver enhanced PbS QDs in the kidney. Bar: 20 μm.

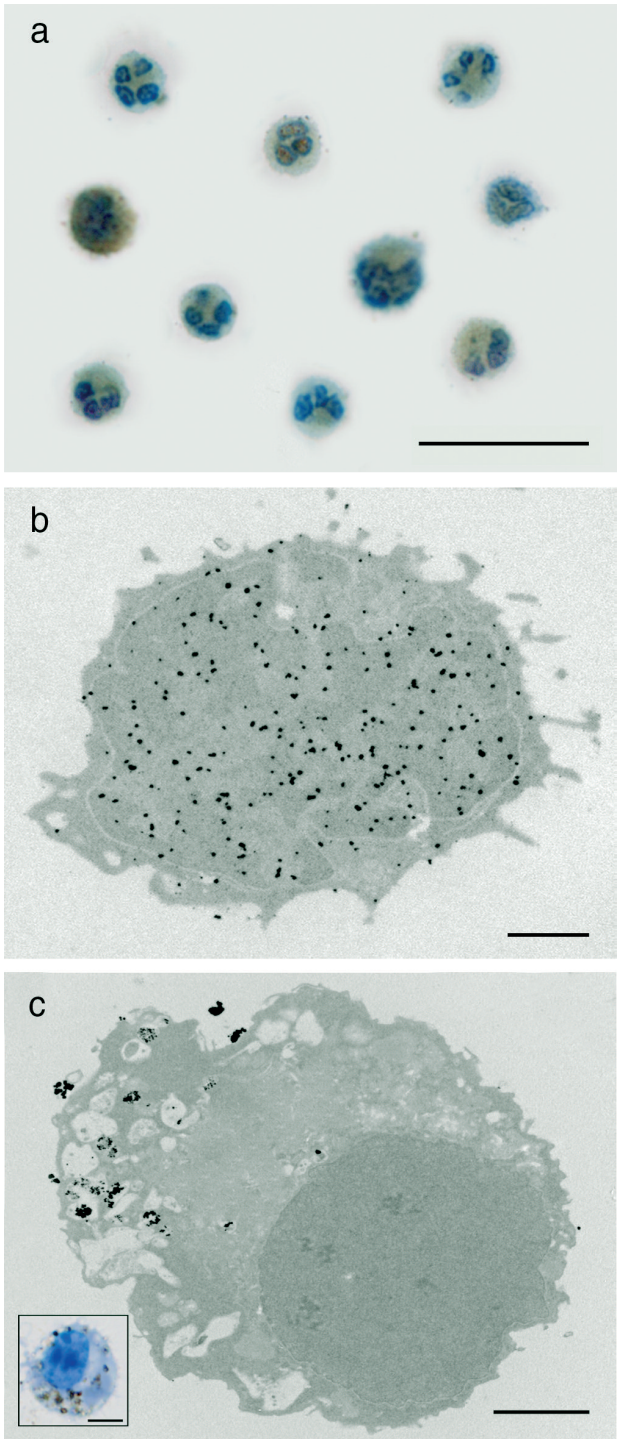

Fig. 2. Light micrograph of AMG silver enhanced leukocytes filled with nanogold. The translocating molecule HIV-Tat peptide was used as carrier. Note that the picture is a mosaic, composed of leukocytes from a smear using an image processing program. Bar: 20 μm. **b.** Electron micrograph showing silver enhanced Gold-Tat in all cellular compartments. Bar: 2 μm. **c.** The "Qtracker cell labeling kit" composed of CdSe encapsulated in a ZnS shell conjugated to a membrane translocating molecule (details from the manufacturer are not available). These QDs were found to be localized in lysosome-like organelles of the j774 macrophage. Bar: 1 μm. Insert: QD labeled leukocyte at light microscopical level. Bar: 10 μm.

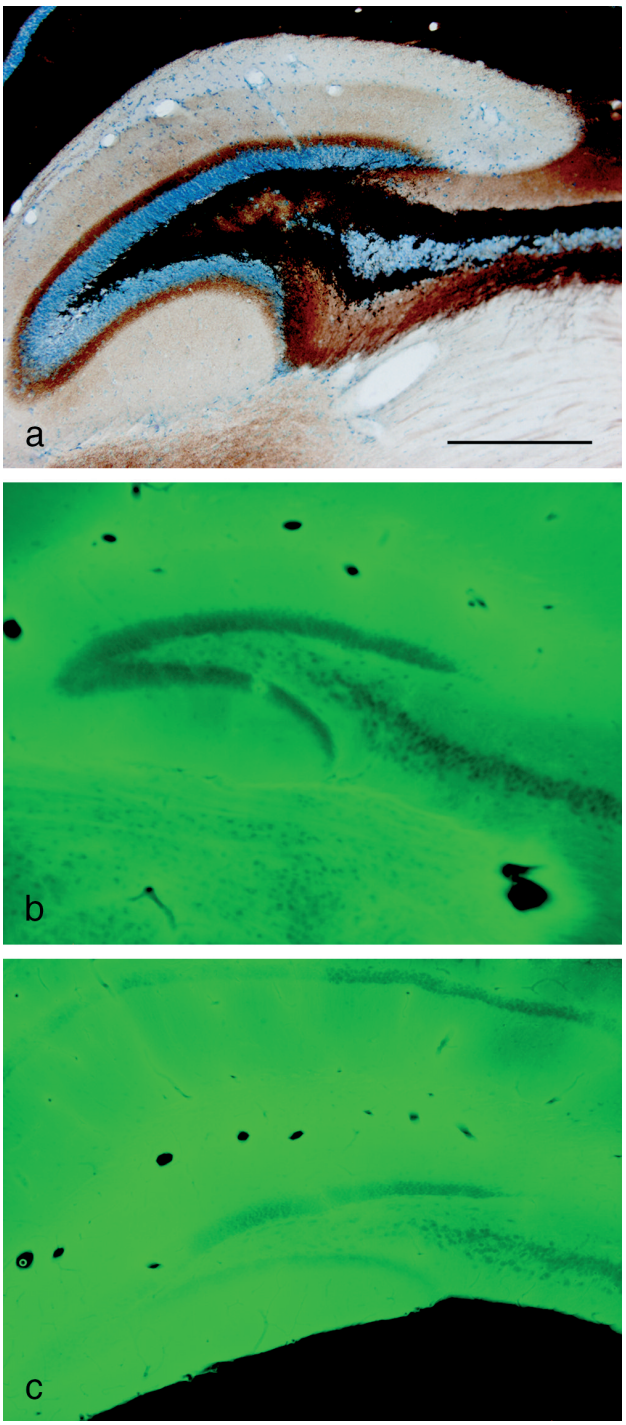

Fig. 3.a. BALB/Ca mouse. Cryo-section, 30 μm, subjected to zinc-selenium autometallography, $ZnSe^{AMG}$. The *in vivo* created zinc-selenium quantum dots are abundantly present in synaptic vesicles of the zinc-enriched (ZEN) terminals of the hippocampus. The difference in staining from yellow to black is a result of the sizes and amounts of the AMG grains, and these again are dependent on the amounts of ZnSe QDs in the individual ZEN terminals. Bar: 300 μm. **b.** Section from the same animal as in figure 3a, but not AMG silver enhanced. The in vivo created ZnSe quantum dots cannot be detected in the fluorescence microscope (Fig. 3c). Bar: 300 μm. **c.** Fluorescence micrograph of blank control. Bar: 300 μm.

visualized with autometallography (Figs. 1, 2, 3a). In semithin Epon sections the AMG silver enhanced QDs were observed in high magnification LM pictures (Fig. 2c), and on ultrathin sections the exact ultrastructural location was visualized in the electron microscope (Fig. 2b,c).

All three tested, commercially available QDs (i.e. CdSe, CdSe/ZnS (shell ZnS, core CdSe) and Pb-S) were found in the epithelial cells lining the small intestinal villi (Fig. 1a). Of these only PbS QDs were found to cluster in the tubular cells of the kidneys (Fig. 1b).

In *in vitro* cultured cells (the macrophage J774 cell line) exposed to QDs coated with membrane translocating molecules (MTM) the particles were AMG traced at light and ultrastructural levels (Fig. 2c). Cells exposed to CdSe QDs adhered to an unknown MTM (name not available from manufacturer) and showed staining of the cytoplasm only (Fig. 2c). The silver enhanced QDs were found to be localized in lysosome like organelles in the electron microscope (Fig. 2c) implying that the QDs were most likely phagocytosed. The QDs were never observed in other cell organelles including nuclei.

Leukocytes filled with nanogold, introduced into the cells by the MTM HIV-Tat peptide, exhibited a brownish tint at LM levels caused by a multitude of AMG enhanced gold nanoparticles (Fig. 2a,b). Electron micrographs revealed that the silver enhanced quantum dots had penetrated into all cellular compartments of the leukocytes, including nuclei (Fig. 2b).

The *in vivo* and *in vitro* created Zn-Se (Fig. 3b) and Zn-S QDs, did not give usable fluorescence signals as it is not possible to distinguish the faint glow from the auto-fluorescence signal of the blank controls (Fig. 3c). In contrast AMG silver enhancement of the same QDs resulted in a highly laminated and distinct pattern that can be analyzed at both LM (Fig. 3a) and EM levels.

Discussion

Our data support the notion that the autometallographic technique is manifold more sensitive than quantum fluorescence. Additionally, the AMG approach has the benefit of allowing QDs to be traced at high LM magnifications (Fig. 2c) as well as ultrastructurally.

The lack of fluorescence signals in brains subjected to selenide and sulphide ions could be due to the small size of the QDs and/or low concentrations. Another explanation could be that the in vivo created QDs do not exhibit the fluorescence characteristic of the synthetic QDs (as they are protected from the water by a coating, *vide supra*).

The reason why the different commercial semiconductor quantum dots cluster in the cells instead of being translocated into the cells as announced by the company we do not know. However, as the goal of the present study was to point to the fact that QDs can be AMG silver enhanced we have done nothing to find out why the product did not function as expected; the problem could even be in our laboratory's handling of the product.

The idea of gold tagging isolated cells was partly based on the finding that exposure to the membrane translocating molecules ("Tat peptides") tagged with magnetic iron nanoparticles causes the cells to be loaded with iron nanoparticles (Josephson et al., 1999). This approach enables the researchers to re-isolate, by way of magnetic field separation, the nanoparticle loaded cells and their progeny after they have been introduced into an organism or a cell culture (Josephson et al., 1999). The benefit of using gold nanoparticles or gilded iron particles instead of iron particles is that gold particles 1) seem to be nontoxic to the loaded cells, and 2) can be traced with AMG silver enhancement in tissue sections from biopsies / autopsies (Danscher 2003).

In conclusion, the autometallographic technique offers an alternative way of visualizing QDs, and the possibility of tracking MTM molecules with QDs might turn out to be a valuable tool in future studies of tissue compatibility, the embryological origin of tissue elements, and the fate of injected stem cells.

Acknowledgements. The authors gratefully acknowledge the skillful laboratory technical assistance of Ms D. Jensen, Ms L. Munkøe and Ms M. Sand. Special thanks to Ms K. Wiedemann for her high-quality, professional work on the manuscript and to Mr. A. Meier for his excellent photographic input. Skejby Sygehus, Aarhus University Hospital, Denmark is gratefully acknowledged for providing the buffy coat leucocytes. The study was supported by The Augustinus Foundation, The Aase and Ejnar Danielsen Foundation, The Aarhus University Research Foundation, The Health Science Faculty, The Beckett Foundation, The Danish Medical Association Research Fund, and Direktør Jacob Madsen og Hustrus Fond.

References

Alivisatos P. (2004). The use of nanocrystals in biological detection. Nat. Biotechnol. 22, 47-52.

Banin U. and Millo O. (2003). Tunneling and optical spectroscopy of semiconductor nanocrystals. Annu. Rev. Phys. Chem. 54, 465-92.

Bentolila L.A. and Weiss S (2003). Biological quantum dots go live. Physics World 3, 23–24.

Bruchez M. Jr., Moronne M., Gin P., Weiss S. and Alivisatos A.P. (1998). Semiconductor nanocrystals as fluorescent biological labels. Science 281, 2013-2016.

Chan W.C., Maxwell D.J., Gao X., Bailey R.E., Han M. and Nie S.W. (2002). Luminescent quantum dots for multiplexed biological detection and imaging. Curr. Opin. Biotechnol. 13, 40-46.

Dahan M. (2005). From analog to digital: exploring cell dynamics with single quantum dots. Histochem. Cell Biol. 125, 451-416.

Dahan M., Levi S., Luccardini C., Rostaing P., Riveau B. and Triller A. (2003). Diffusion dynamics of glycine receptors revealed by single-quantum dot tracking. Science 302, 442-445.

Danscher G. (1981). Light and electron microscopic localization of silver in biological tissue. Histochemistry 71, 177-186.

Danscher G. (1982). Exogenous selenium in the brain. A histochemical

technique for light and electron microscopical localization of catalytic selenium bonds. Histochemistry 76, 281-293.

Danscher G. (2003). Organometallic probe. Patent application; International application number PCT/DK02/00486; International publication number WO 03/006681.

Danscher G. and Stoltenberg M. (2005). Zinc-specific autometallographic in vivo selenium methods: tracing of zinc-enriched (ZEN) terminals, ZEN pathways, and pools of zinc ions in a multitude of other ZEN cells. J. Histochem. Cytochem. 53, 141-153.

Danscher G. and Stoltenberg M. (2006). Autometallography (AMG). Prog. Histochem. Cytochem. 41, 57-139.

Danscher G., Hacker G.W., Hauser-Kronberger C. and Grimelius L. (1995). Trends in autometallographic silver amplification of colloidal gold particles. In: Immunogold-Silver Staining. Principles, Methods, and Applications. Hayat M.A. (ed). CRC Press. Boca Raton. pp 11-18.

Dubertret B., Skourides P., Norris D.J., Noireaux V., Brivanlou A.H. and Libchaber A. (2002). In vivo imaging of quantum dots encapsulated in phospholipid micelles. Science 298, 1759-1762.

Empedocles S. and Bawendi M. (1999). Spectroscopy of single CdSe nanocrystallites. Acc. Chem. Res. 32, 389-396.

Fu A., Gu W., Larabell C. and Alivisatos A.P. (2005). Semiconductor nanocrystals for biological imaging. Curr. Opin. Neurobiol. 15, 568-575.

Gao X., Cui Y., Levenson R.M., Chung L.W. and Nie S. (2004). In vivo cancer targeting and imaging with semiconductor quantum dots. Nat. Biotechnol. 22, 969-976.

Hardman R. (2006). A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors. Environ. Health. Perspect. 114, 165-172.

Josephson L., Tung C.H., Moore A. and Weissleder R. (1999). High-efficiency intracellular magnetic labeling with novel superparamagnetic-Tat peptide conjugates. Bioconjug. Chem. 10, 186-191.

Medintz I.L., Uyeda H.T., Goldman E.R. and Mattoussi H. (2005). Quantum dot bioconjugates for imaging, labelling and sensing. Nat. Mater. 4, 435-446.

Michalet X., Pinaud F.F., Bentolila L.A., Tsay J.M., Doose S., Li J.J., Sundaresan G., Wu A.M., Gambhir S.S. and Weiss S. (2005). Quantum dots for live cells, in vivo imaging, and diagnostics. Science 307, 538-544.

Rieger S., Kulkarni R.P., Darcy D., Fraser S.E. and Koster R.W. (2005). Quantum dots are powerful multipurpose vital labeling agents in zebrafish embryos. Dev. Dyn. 234, 670-681.

Stroh M., Zimmer J.P., Duda D.G., Levchenko T.S., Cohen K.S., Brown E.B., Scadden D.T., Torchilin V.P., Bawendi M.G., Fukumura D. and Jain R.K. (2005). Quantum dots spectrally distinguish multiple species within the tumor milieu in vivo. Nat. Med. 11, 678-682.

Timm F. (1958). Zur Histochemie der Schwermetalle. Das Sulfid-Silberverfahren. Dtsch. Z. Gerichtl. Med. 46, 706-711.

Warburton R.J., Schaflein C., Haft D., Bickel F., Lorke A., Karrai K., Garcia J.M., Schoenfeld W. and Petroff P.M. (2000). Optical emission from a charge-tunable quantum ring. Nature 405, 926-929.

Wu X., Liu H., Liu J., Haley K.N., Treadway J.A., Larson J.P., Ge N., Peale F. and Bruchez M.P. (2003). Immunofluorescent labeling of cancer marker Her2 and other cellular targets with semiconductor quantum dots. Nat. Biotechnol. 21, 41-46.

Accepted December 13, 2006