Cadmium Telluride Quantum Dots as a Fluorescence Marker for Adipose Tissue Grafts

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Abstract: Plastic and reconstructive surgeons increasingly apply adipose tissue grafting in a clinical setting, although the anticipation of graft survival is insecure. There are only few tools for tracking transplanted fat grafts in vivo.

Murine adipose tissue clusters were incubated with negatively charged, mercaptoproprionic acid–coated cadmium telluride quantum dots (QDs) emitting in the dark red or near infrared. The intracellular localization of QDs was studied by confocal laser scanning microscopy.

As a result, the adipose tissue clusters showed a proportional increase in fluorescence with increasing concentrations (1, 10, 16, 30, 50 nM) of cadmium telluride QDs. Laser scanning microscopy demonstrated a membrane bound localization of QDs. Vacuoles and cell nuclei of adipocytes were spared by QDs. We conclude that QDs were for the first time proven intracellular in adult adipocytes and demonstrate a strong fluorescence signal. Therefore, they may play an essential role for in vivo tracking of fat grafts.

Key Words: quantum dots, qdots, adipocyte, tracing, tracer, fat graft, lipostructure, adipose tissue, fluorescence marker, bioluminescence

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In the last decade, there has been a renaissance of fat transplantation. It has become a frequently applied method for plastic and reconstructive surgeons for the treatment of tissue defects or functional deficits like in vocal folds.[1–4]

The clinical use of the method by far outnumbers the scientific knowledge, which is currently evolving.[1,5,6] A large focus has been laid lately on progenitor cells to enhance the clinical outcome and overcome unforeseeable results with transplantation of adult fat cluster.[7,8] The clinical reality that remains so far is the transplantation of adult adipose tissue clusters.[9]

Only few experimental models resemble the adipose tissue cluster transplantation. Adult adipocytes cannot be treated as other primary cell types in vitro. Therefore, we used methods which mirror and refer to the clinical techniques for fat transplantation.

Semiconductor nanocrystals (colloidally grown quantum dots [QDs]) are solution-processed nanoparticles in the size of usually less than 10 nm, which exhibit remarkable fluorescence properties.[10–13] Quantum dots are currently used for cell tracking in various cell and tumor cell types and can also be traced in vivo,[12,13] as demonstrated below.

The aim of the study was to localize QDs in adipose tissue clusters as a premise for a potential adipose tissue labeling. Quantum dots are evaluated in the following experiments as markers and tracers for adipocyte cluster transplants.

METHODS

Materials and Animals

The cadmium telluride (CdTe) QDs were synthesized in water as previously described.[14] They exhibit a net negative surface charge due to capping with mercaptoproprionic acid.[15] Dark red emitting QDs (denoted as QD675, with emission peak at 675 nm when excited at 633 nm) with an average size of 4 nm were used in microscopy studies, while near-infrared emitting QDs denoted as QD770 (emission peat at 770 nm when excited at 740 nm) with a size of 6.5 nm similar as in Zintchenko et al[16] were used for macroscopic imaging of labeled fat clusters. QD770 did not easily allow detection with laser microscopy while showing a suitable spectrum for near-infrared in vivo tracing, whereas the QD675 profile seemed more appropriate for laser microscopy studies.

NaCl 0.9% was purchased from B. Braun (Melsungen, Germany) and used as a short-time storage solution and for incubation experiments. 4′,6-Diamidin-2-phenylindol (DAPI) was purchased from Sigma (Munich, Germany) and used as a 1-mg/mL stock solution in water. Six-well cell culture plates were from TPP (Transadigen, Switzerland).

Balb/c mice (Janvier, Le Genest-St-Isle, France) were housed in individually vented cages (type 2 L) with free access to mouse chow and water. Animals were allowed to accommodate to the standard conditions of the animal facility for at least 1 week before the start of experiments.

All experiments were performed according to the guiding principles for research involving animals and the German legislation on protection of animals. Approval was obtained from the local governmental animal care committee.

Adipocyte Cluster Isolation

Adipocyte clusters were harvested from the inguinal, subcutaneous, and abdominal region of Balb/c mice and mechanically transected with scalpels. Special care was taken to avoid crushing the adipocyte clusters during the harvesting process. The collected adipocyte clusters were diluted in NaCl 0.9% on 6-well cell culture plates. Adipocyte clusters weighed between 50 and 100 mg. The differences are explained due to the mechanical dissection of the fat and resulted in a cluster size, that was comparable to the clinical situation and potentially injectable in vivo for further transplantation experiments.

Because adipocytes did not adhere on cell culture dishes, there was inevitable cell-air contact. Adipocytes were immediately used for incubation experiments without collagenase digestion or any other

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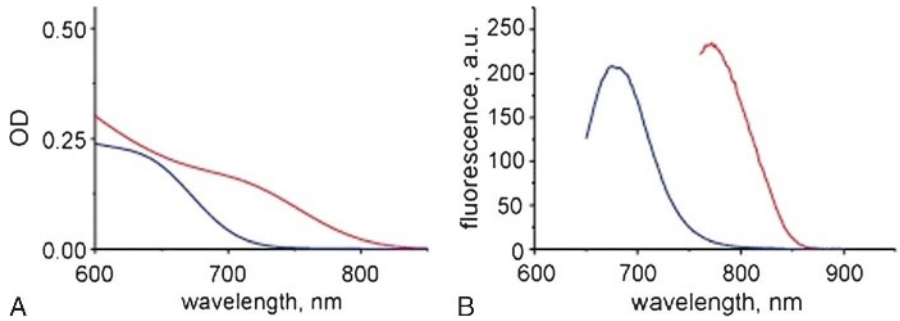

FIGURE 1. Absorption (A) and emission (B) spectra of QD675 (black line) and QD770 (grey line). The excitation wavelength was 633 nm for QD675 and 740 nm for QD770.

type of further processing. A reliable determination of vitality would have been arbitrary for the whole clusters and was therefore not monitored. In particular, neither cell culture medium nor fetal calf serum were applied.

Control adipocyte clusters were devitalized within 1.5-mL tubes by being heated up to 80°C in a block heater (Eppendorf, Hamburg, Germany) for 45 minutes and subsequently cooled down to 37°C.

Laser-Scanning Microscopy

For laser-scanning microscopy studies, freshly isolated adipocyte clusters, were used immediately after 1-hour incubation at 37°C with 22 µmol/L QD675 in NaCl 0.9%. The high concentration was chosen to ensure a complete saturation in the labeling process and does not represent a suitable dosage for in vivo experiments. As a control group, adipocyte clusters without QDs incubation were used. Labeled and unlabeled adipocyte clusters were placed into Lab-Tek 8 chambered cover glasses (Nalge Nunc, Naperville, Ill). Confocal laser-scanning microscopy was carried out with a LSM 710 META microscope (Carl Zeiss, Jena, Germany) equipped with gas lasers emitting lines at 364 nm (argon), 488 nm (argon), and 633 nm (helium/neon laser). Pictures were taken with a plan-Apochromat 63×/1.40 Oil differential interference contrast (DIC) objective.

For DNA and consecutive cell nucleus marking, DAPI was used. Fluorescence detection of QD675 CdTe QDs was achieved with a 650-nm longpass filter and laser excitation at 633 nm. 4′,6-Diamidin-2-phenylindol was spotted with a 385- to 470-nm bandpass filter and excitation at 364 nm. Data were acquired and analyzed with AxioVision 4.6 software, LSM image browser software, and ZEN 2009 LE Software (Carl Zeiss, Jena, Germany).

Near-Infrared Imaging

Freshly harvested adipocyte clusters were kept in 6-well plates and incubated in ascending concentrations of QD770 for 1 hour at 37°C (n = 5), followed by 2 washing steps with NaCl 0.9%. Near-infrared

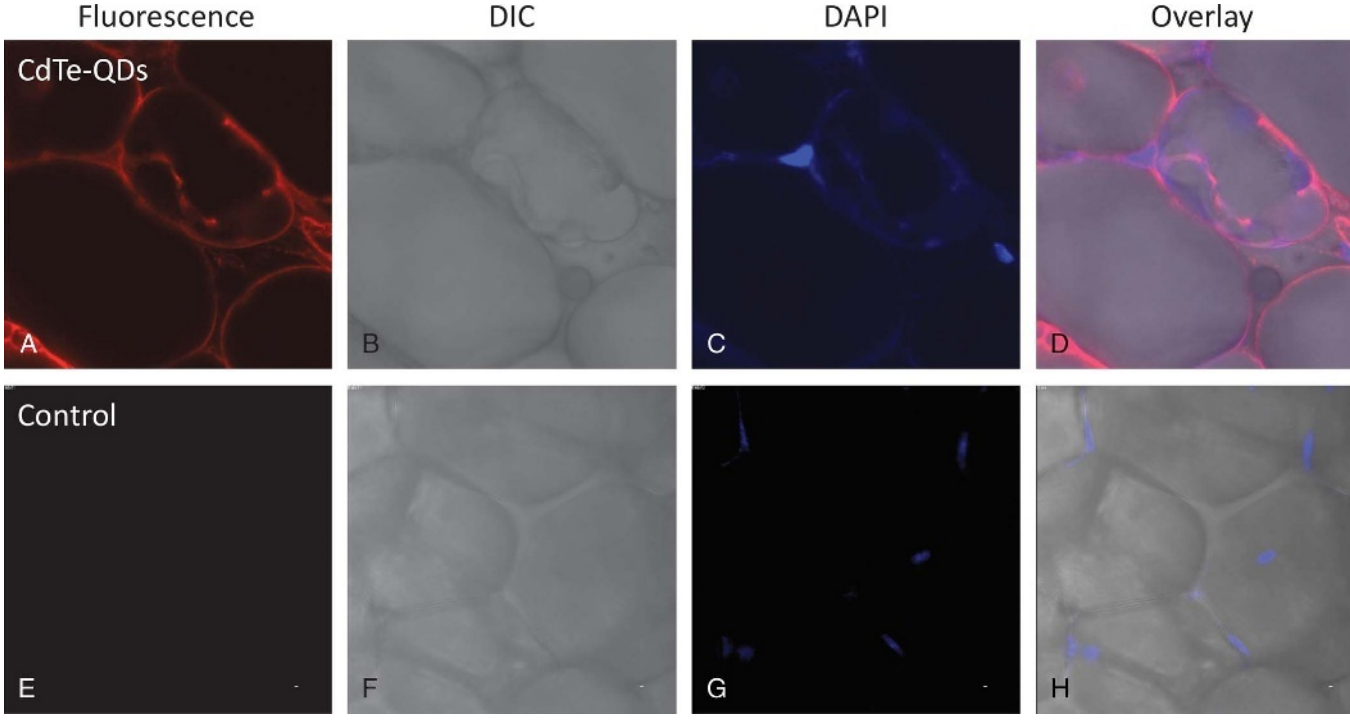

FIGURE 2. Confocal laser-scanning microscopy. A–D, Adipocyte clusters, incubated with QD675. E–H, Control: Adipocyte clusters without QDs treatment for exclusion of autofluorescence. Mode: plane 2D. Field of view is 112.5 × 112.5 µm. Fluorescence was encoded in red (A, E): LP 650 nm, Excitation with 633 nm. DIC (B, F) (differential interference contrast). DAPI was encoded in blue (C, G): BP 385–470 nm, excitation with 364 nm. Overlay image with all three channels (D, H). LP, longpass; BP, bandpass.

imaging was immediately carried out with an IVIS® Lumina Imaging System (Xenogen, Caliper Life Science, Rüsselheim, Germany) as previously described.[16] Samples were illuminated with a halogen lamp and a filter combination suitable for near-infrared imaging (excitation filter, 710–760 nm; emission filter, 810–875 nm) with 1-second exposure time. Reflected light pictures were taken by illumination with 4 white light-emitting diodes. Signal processing and imaging was performed with the Living Image Software 3.0 (Caliper Life Science). Acquired fluorescence signals were automatically background corrected by the software from the signal of the surrounding area and overlaid onto the reflected light image.

RESULTS

Ex Vivo Labeling of Adipocyte Clusters With QDs

The emission maxima for QDs when excited at 633 and 740 nm, respectively, are shown in Figure 1. Immediately after isolation, adipocyte clusters were incubated with highly concentrated aqueous QDs solutions (22 μmol/L) for 1 hour. No microscopically noticeable difference in cell morphology or vitality was noted after the incubation. When using devitalized adipocyte clusters after heat treatment, QDs showed strong adhesion artifacts resulting in the elevated fluorescence signal. Similar adhesion artifacts were also seen in our experiments with clusters devitalized with chemical agents, that is, alcohol derivatives and other chemical agents that are frequently used for induction of cell death (data not shown). Therefore, these substances could not be used for creating control groups for incubation experiments with QDs used here.

Intracellular Distribution of QDs and Analysis of Autofluorescence

Laser-scanning microscopy was carried out immediately after incubation with QD675 as described above. The use of chambered cover glass allowed direct analysis for viable material on an inverted microscope using an oil immersion objective (Fig. 2). QD675-labeled adipocyte clusters (Figs. 2A–D) and control adipocyte clusters without QD incubation (Figs. 2E–H) were analyzed. For the control group, filter settings were unchanged. The field of view was 112.5 × 112.5 μm. The first channel (software color coding in red) showed fluorescence of QDs with a longpass filter of 650 nm and an excitation wavelength of 633 nm. Incubated adipocyte clusters showed a strong fluorescence signal (Fig. 2A), whereas controls without QDs did not reveal a fluorescence signal (Fig. 2E). Therefore, autofluorescence and a misreading of a tracking signal in the monitored wavelength can be excluded.

Channel 2 (Figs. 2B, F) represented the DIC image of the adipocyte clusters. 4′,6-Diamidin-2-phenylindol documented cell nuclei and was colored blue in channel 3 (Figs. 2C, G). For this channel, a bandpass filter from 385 to 470 nm was used with an excitation ($\lambda = 364$ nm). The fused overlay images show all 3 channels (Figs. 2D, H).

3D Localization of QDs

To localize the negatively charged CdTe QDs within adipocytes, laser-scanning microscopy was used in plane (2D) and stack (3D) mode. Excitation wavelength and filter settings were used as described above.

Figure 3 demonstrates a plane overlay image of QD fluorescence (red), differential interface contrast and DAPI (blue). The QD fluorescence can be observed in the extracellular matrix, within fat cells, and on the cell membranes. The fat vacuoles were apparently not penetrated by QDs. However, there was an affinity of the negatively charged QDs for vacuole and cell membranes, due to a fluorescence signal enhanced on the membrane borders. At high magnification and with an optical section thickness of 1.6 μm, there was no overlap in QD signal with the DAPI marked cell nuclei observed, suggesting no nuclear uptake of QDs (Fig. 3, insert).

The stack laser-scanning mode was used for 3D examination of adipocyte clusters (Fig. 4). Filter settings and excitation were kept as described above. Field of view was in 3 dimensions: $x = 318.2$ μm; $y = 318.2$ μm; $z = 23.4$ μm. In the orthogonal view (Fig. 4A), the observation of intracellular accumulation of QD fluorescence within the adipocyte clusters can be validated. A 3D reconstruction like in Figure 4B can be generated from stack mode data sets and creates a rewarding microanatomical view.

Labeling of Fat Cluster With Near-Infrared Emitting QDs

Serial incubation of adipocyte clusters with increasing concentrations of near-infrared emitting QD770 was followed by analysis with an IVIS® Lumina Imaging System. Excitation filters were set to 710 to 760 nm, emission filters to 810 to 875 nm (Fig. 5). Fluorescence of adipocyte clusters was quantified by placing defined regions of interest onto the clusters for signal analysis (Fig. 6). Quantitative measurements

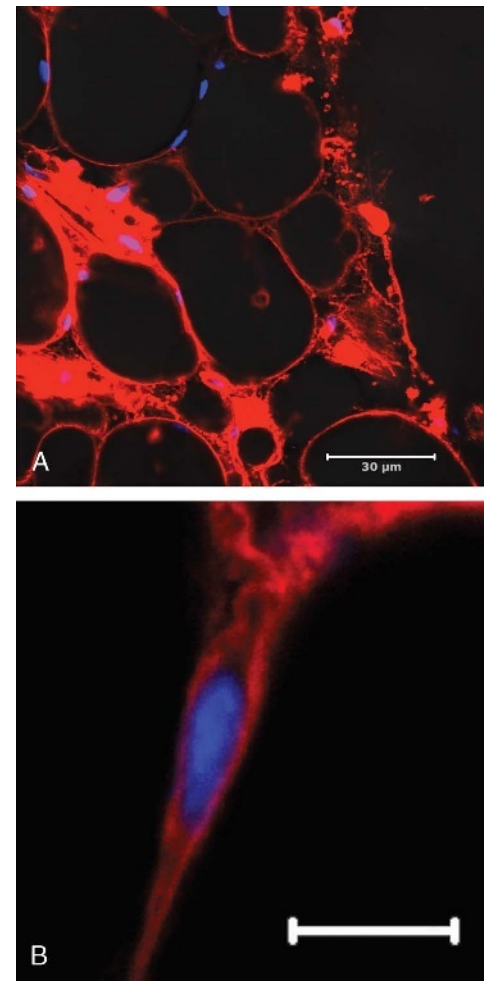

FIGURE 3. A, QD675 demonstrated fluorescence signal at the outer cell and vacuolar membranes (red) after incubation of micro fat clusters. Fat vacuoles were not penetrated from the QDs. DAPI (coded in blue) was used for nucleus detection. Excitation with 633 and 364 nm, LP filter with 650 nm and BP filter between 385 and 470 nm was used. B, high magnification of QD675 labeled fat cluster: red: QD signal, blue: DAPI signal, optical section thickness 1.6 μm; Scale bar, 6.3 μm.

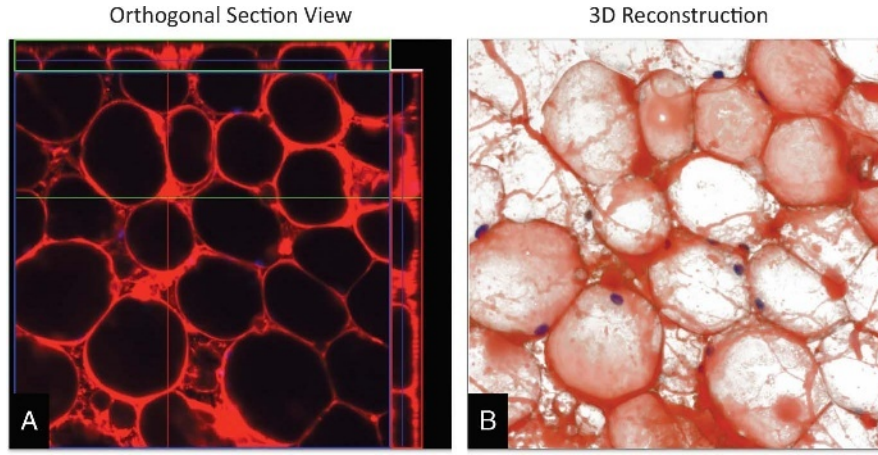

FIGURE 4. In the stack scanning mode 3D studies can be performed. Fluorescence marking (red) was found intracellular in all dimensions of the adipocyte cluster sections. DAPI (blue) was used as a nucleus marker. Field of view: $x = 318.2$ µm; $y = 318.2$ µm; $z = 23.4$ µm. Stack images can be viewed as an orthogonal view (A) or a 3D reconstruction (B).

revealed a progression of the fluorescence with an increase in QDs concentration (1, 10, 16, 30, 50 nmol/L).

DISCUSSION

Adipose tissue clusters are used frequently as microtransplants from plastic and reconstructive surgeons under terms like lipofilling or autologous fat grafting.[9,17] Fat grafting is used for aesthetic and functional indications.[4] However, models for reliable prediction of outcomes both in vitro and in vivo are still lacking from these clinically established methods.[5,18] Handling of adult adipocytes in cell culture is known to be difficult. Many authors focus their research on progenitor or stem cells, due to the promising capabilities of these cell types[7,19,20] and refrain from research with conventional lipoaspirates. As a consequence, the frequent use of lipoaspirate grafting in the clinical setting is not supported by extensive experimental data so far.

Several tracing dyes or other fluorescent compounds were tested for lipoaspirates. Rieck and Schlaak[18] used the lipophilic fluorescence dye PKH26 for staining of a cell suspension, which was obtained by collagenase incubation of adipose rat tissue. The cell suspension was transplanted and after 6 months explanted and histomorphologically examined. This method was also used for the transplantation of preadipocytes.[21]

Several substances for fluorescence tracking of human preadipocytes were proposed in vitro by Hemmrich et al.[22] carboxyfluorescein diacetate succinimidyl ester, CM-Cil, and PKH26 showed a remaining fluorescence of roughly 4% to 6% after 35 days in vitro.

Semiconductor QDs offer strong and stable emission for fluorescent labeling of living tissues.[10,13,16,23] There have been several attempts for QD ligand binding to enhance target specifity.[16,24,25] Quantum dots were used as tracing particles for adipose tissue-derived stem cells (ASCs) by Yukawa et al.[26] CdSe/ZnS-core/shell nanocrystals were used for ASC labeling and a cationic liposome has been used for delivery into cells. In addition, the group published a method including a cell penetrating peptide (R8) for delivery into ASCs, where the fluorescence intensity of QDs was maintained for at least 2 weeks using QD655.[27] For our experiments, we used CdTe core-only QDs, which were directly synthesized in water. Their surface was coated as synthesized with mercaptoproprionic acid, which stabilizes the particles and leaves a net negative surface charge due to the

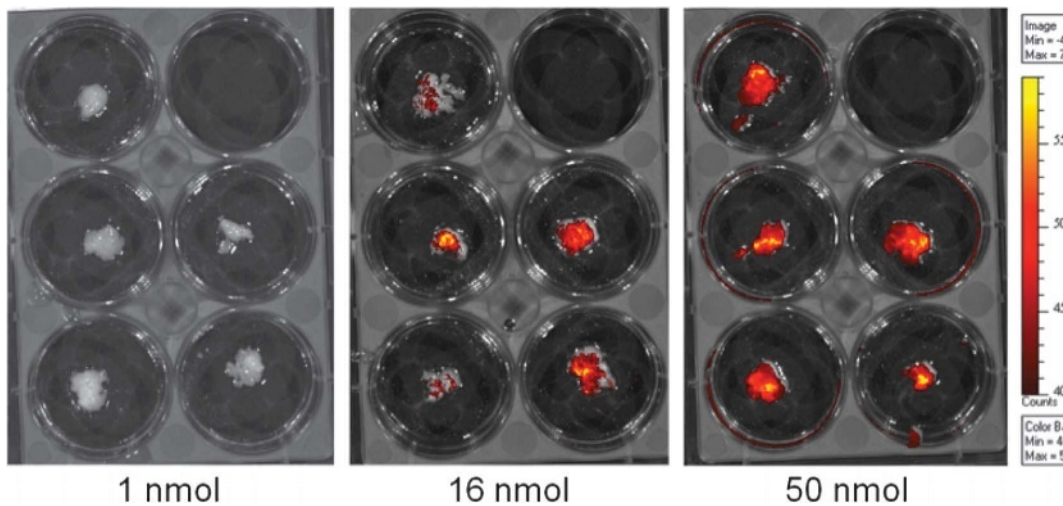

FIGURE 5. Near-infrared imaging of QD770 incubated with adipocyte clusters of Balb/c mice in ascending concentrations. Excitation filter was set to 710–760 nm, emission filter 810 to 875 nm. Testing was performed in the IVIS® chamber; fluorescence signal was fused online with photo-optical image with Living Image 3.0 software.

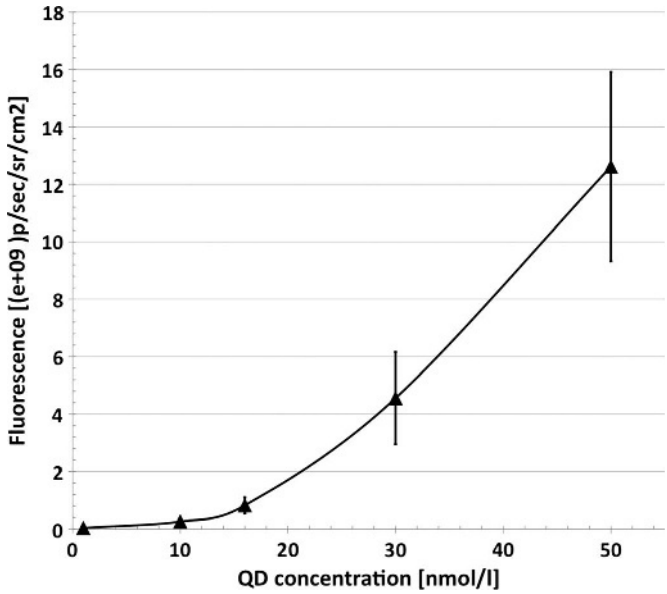

FIGURE 6. AC were incubated for 1 hour with QD770 in increased concentrations and showed a proportional increase in fluorescence. AC, adipocyte clusters.

presence of carboxyl groups. For the first time, intracellular distribution of QDs within adipocytes was demonstrated. In our study, we did not apply any enhancer compounds to promote cellular uptake, although one would assume lack of internalization due to charge repulsion between the negatively charged QDs and the negatively charged phospholipids within the cell membrane. Recently, it was reported that both QDs with positive and negative surface charge are decorated by serum proteins when incubated with undiluted fetal calf serum in vitro.[28] In our experiments, we assume a similar mechanism, where residual proteins, for example, HDL, LDL, or albumin from serum or from within the extracellular matrix of adipocyte clusters, decorate QDs and allow their internalization via a "piggy back" mechanism into the cell. Once inside the cell, QDs can then be incorporated into various intracellular membranes. We did not observe any QD fluorescence within the cell nuclei, which may be related to tight size restrictions: similar CdTe QDs with a size of 3.8 nm have been reportedly excluded from the nucleus,[15] whereas the diameter of our QD675, even not taking in to account their protein decoration, is 4 nm.

Because the core of commonly used QDs contains heavy metals, like cadmium in CdTe QDs used in our experiments, cell toxicity is a major concern. CdTe QDs reportedly showed cytotoxic properties to a breast cancer cell line, which were not dose dependent to the intracellular Cd^{2+} levels.[29] Yukawa et al[26] demonstrated cytotoxicity of core-shell CdSe/ZnS QDs to ASCs, when transfected with QD655-lipofectamine more than 2 nM. Other groups observed no histological changes with core-shell CdSe(0.25)Te(0.75)/CdS QDs in major organs, when administered in a mouse model, even at high doses.[30] High-dose QD concentration, as presented in our confocal microscopy studies, would certainly induce a long-term or even acute cytotoxicity. At the same time, we recognized no acute signs of cell decay during the period of laser-scanning microscopy. High concentrations of QDs used here were chosen for the purpose of facilitated intracellular localization. For tracking purposes, the lowest possible concentration of QDs should be applied. Further testing concerning toxicity and dosing of QDs would be necessary for long-term in vivo use, including leaking and in vivo pharmacodynamics studies.

Using fat clusters instead of single cells enable us to preserve the intact fat compound without destroying surface proteins, or other cell interaction features, as seen by single cell preparations. The clinical reality of transplanted fat clusters was replicated closely in our model. With the presented method, we demonstrated for the first time an intracytoplasmatic fluorescence marking of adipose tissue with deep-red emitting and near-infrared emitting CdTe QDs. This technique appears suitable to expand the arsenal of fat grafting research.

The QDs marked fat tissue cluster could be transplanted, grafted, and traced in vivo by fluorescence tracking systems like the IVIS® Lumina Imaging System. Other applications are micro 3D reconstruction of adipose tissues or grafts for observation.

We conclude that labeling of adipose tissue clusters with QDs has been demonstrated for the first time. Negatively charged CdTe QDs with a diameter of 4 nm and an emission maximum of 675 nm (QD675) were associated with cell membranes including the vacuole membrane, but did not penetrate the fat vacuoles and cell nuclei. Larger (6.5 nm) QDs with an emission maximum of 770 nm (QD770) also labeled fat clusters and could be conveniently traced with a near-infrared fluorescence imaging system. We present a viable tool for adipose tissue fluorescence reconstruction in 3D, as well as for fat graft tracing in vivo.

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