Published in final edited form as:

Invest Ophthalmol Vis Sci. 2015 May 1; 56(5): 3051–3059. doi:10.1167/iovs.14-15973.

Quantum dot labeling and tracking of cultured limbal epithelial cell transplants *in-vitro*

Miss Nuria Genicio, BSc^{1,2}, Dr Juan Gallo Paramo, PhD^{3,4}, and Mr Alex J. Shortt, MB BCh MSc PhD FRCOphth^{1,2,5}

¹Department of Ocular Biology and Therapeutics, UCL Institute of Ophthalmology, Bath Street, London, EC1V 9EL

²NIHR Biomedical Research Centre, Moorfields Eye Hospital and UCL Institute of Ophthalmology, 162 City Road, London, EC1V 2PD

³Comprehensive Cancer Imaging Centre. Imperial College, South Kensington, London, London SW7 2AZ

⁴Department of Chemistry, Imperial College London, South Kensington, London, SW7 2AZ

⁵Cornea and External Disease Service, Moorfields Eye Hospital NHS Foundation Trust, 162 City Road, London, EC1V 2PD

Abstract

PURPOSE—Cultured human limbal epithelial cells (HLEC) have shown promise in the treatment of limbal stem cell deficiency but little is known about their survival, behaviour and long-term fate post transplantation. The aim of this research was to evaluate, in-vitro, quantum dot (QDot) technology as a tool for tracking transplanted HLEC.

METHODS—In-vitro cultured HLEC were labeled with Qdot nanocrystals. Toxicity was assessed using live-dead assays. The effect on HLEC function was assessed using colony forming efficiency assays and expression of CK3, P63alpha and ABCG2. Sheets of cultured HLEC labeled with Qdot nanocrystals were transplanted onto decellularised human corneo-scleral rims in an organ culture model and observed to investigate the behaviour of transplanted cells.

RESULTS—Qdot labeling had no detrimental effect on HLEC viability or function in-vitro. Proliferation resulted in a gradual reduction in Qdot signal but sufficient signal was present to allow tracking of cells through multiple generations. Cells labeled with Qdots could be reliably detected and observed using confocal microscopy for at least 2 weeks post transplantation in our organ culture model. In addition it was possible to label and observe epithelial cells in intact human corneas using the Rostock corneal module adapted for use with the Heidelberg HRA.

Corresponding author: Mr Alex J Shortt MB BCh MSc PhD FRCOphth Wellcome Trust Fellow & Honorary Consultant Ophthalmologist in Cornea and External Disease NIHR Biomedical Research Centre for Ophthalmology Moorfields Eye Hospital NHS Foundation Trust and UCL Institute of Ophthalmology Phone: +44(0)207 566 2320 Mobile: +44(0)79 772 44923 Fax: +44(0)20 7566 2334 a.shortt@ucl.ac.uk.

Meeting presentations: Provisional data from this manuscript was presented as a paper at ARVO 2007

Proprietary interest statement: No author has a proprietary interest in this work and there is no conflict of interest for any author.

CONCLUSIONS—This work demonstrates that Qdots combined with existing clinical equipment could be used to track HLEC for up to 2 weeks post transplantation, however, our model does not permit the assessment of cell labeling beyond 2 weeks. Further characterisation in in-vivo models are required.

Keywords

Stem cell; cornea; quantum dot; cell tracking; cell transplantation; limbal epithelium

Introduction

Transplantation of *ex-vivo* cultured limbal epithelial stem cells (LESC) is an established treatment for human patients with corneal limbal stem cell deficiency (LSCD).[1-3] Studies reporting outcomes of this treatment use a variety of different measures and means of assessment.[2, 4, 5] These include grading of clinical outcomes, impression cytology sampling of the ocular surface with cytokeratin profiling, *in-vivo* confocal microscopy and examination of excised penetrating keratoplasty buttons. These outcome measures provide the evidence base for the effectiveness of this therapy and for its increasing clinical use.[6-9]

One aspect of this cell therapy that cannot be assessed with certainty is the survival and fate of transplanted cells in patients post-treatment. Genotyping using polymerase chain reaction (PCR) technology has been employed to determine the origin of the cells populating the ocular surface post-treatment but this approach is limited by the fact that it is an invasive procedure that can only be used in allogeneic transplants to detect differences in donor and recipient DNA profiles.[7] Another drawback is that the presence of donor DNA on the ocular surface is not necessarily evidence of successful engraftment of functioning donor stem cells. To date, no study has demonstrated direct evidence that the improved clinical appearance of the cornea following this therapy is directly related to the survival and function of transplanted cells. The fate of transplanted cells is unknown and can only be truly understood by tracking and monitoring transplanted cells in real time *in-vivo*.

The tracking of cells *in-vivo* is a vibrant research area and new tools are emerging.[10] One such tool is quantum dot nanotechnology.[11] Quantum dots (Qdots) are tuneable fluorophores, particles that can be efficiently excited at any wavelength shorter than their emission wavelength then re-emit photons at a longer wavelength. Variation of material and size of these semiconductor results in different emission colours (spanning from the UV to the infra-red) but all of which are excitable with a single wavelength.[12] Qdots have been estimated to be up to 20 times brighter and 100 times more stable than standard organic dyes.[12] These unique properties enable them to be used in biomedical applications such as imaging and tracking. The high photo-stability and emission intensity make them an ideal tool for ultrasensitive detection and long-term imaging.[13] The surface chemistry of Qdots dictates how they behave in cell cultures. Biofunctionalization, the coating of quantum dots with biologically active agents, can render Qdots functional, biocompatible, water soluble and safe when interacting with biological systems. Most common and suitable surface coatings are biomolecules such as peptides, sugars and biopolymers. Although several

groups have applied Qdots *in vivo* in animal models they have yet to be utilized in humans. [14]

The aim of this work was to investigate the safety and efficacy of Qdots as a non-invasive method of labeling and tracking mixed populations of human limbal epithelial cells (HLEC) in an organ culture model of this cell therapy.

Methods

Tissue for in-vitro imaging, immunofluorescence and cell culture studies

Cadaveric human corneas with research consent were obtained from the Moorfields Eye Hospital Lions Eye Bank (UK), North Carolina Eye Bank (U.S.A.) and San Diego Eye Bank (U.S.A.). Experiments on human tissue were approved by the local research ethics committee. Cadaveric rabbit corneas were obtained from an abattoir.

Labeling of primary HLEC in culture

Primary HLEC were isolated from cadaveric human donor corneas and cultured on a mitomycin-C (MMC) growth arrested 3T3 feeder layer in corneal epithelial culture medium (CECM) as previously described.[15] Cells were seeded and cultured in Permax 4-well chamber slides (Nunc® Lab-Tek® Chamber Slide™ system, Sigma UK) for 7 to 10 days following cell isolation until macroscopically visible colonies had grown. They were then labeled using a commercially available kit, the Qtracker® 655 Cell Labeling Kit from Molecular Probes / Invitrogen (Q25021MP). This kit employs a custom cell penetrating peptide bound to the surface of Qdots to induce their cellular uptake. The manufacturers' protocol was followed: 4µL each of Qtracker® component A, which comprised the nanocrystals, and component B, which contained the cell penetrating peptide, were mixed in a 2.0mL microcentrifuge tube. This solution was left to stand at room temp for 5 minutes. Serum containing cell culture medium (800µL) was added and the solution was vortexed for 30 seconds to produce the final labeling solution of which 200µL was added to each well of the 4-well chamber slide containing HLEC. Slides containing cells that had been through the labeling process with the exception of the Qdot reagent were used as negative controls. The cells were incubated at 37°C for 60 minutes following which they were washed twice with culture medium.

Imaging of Qdot labeled cells

Cells were imaged using both fluorescence and confocal microscopy. An Olympus digital light/fluorescence microscopy system (Olympus, Japan) was used to capture fluorescence images. A HBO lamp with a 450 – 550nm band pass filter was used for excitation and a 650nm long pass filter for detection. These images were analysed using image analysis software (Soft Imaging System GmbH, Munster, Germany). Confocal images were captured using a Zeiss LSM 510 confocal microscope (Zeiss GmbH, Germany). A 548nm Argon laser was used for excitation and a 650nm long pass filter for detection.

Electron microscopy to confirm the cellular location of Qdots

Samples of HLEC labeled with Qdot were fixed for 2–12 hours with a mixture of 1% paraformaldehyde and 3% glutaraldehyde in 0.1 M sodium cacodylate buffered to pH 7.4 with 0.1 M HCl at 4 °C. Samples were rinsed with three changes of 0.1 M sodium cacodylate (pH 7.4) and transferred to 1.0% aqueous osmium tetroxide for a further 2 hours. Following osmication, samples were dehydrated in ascending alcohols and propylene oxide and infiltrated overnight in a 1:1 mixture of propylene oxide and araldite. Following two 3 hour changes of araldite, specimens were embedded and cured overnight at 60 °C. Semi- and ultrathin sections were cut with an ultracut S microtome (Leica Microsystems GmbH, Wetzlar, Germany) fitted with a diamond knife. Sections for electron microscopy were collected on copper grids and examined either stained in uranyl acetate and lead citrate or unstained in a Jeol 1200 EX transmission electron microscope (Jeol, Tokyo Japan) operating at 80 kV.

Toxicity of Qdot in labeled HLEC

HLEC were cultured in Permax 4-well chamber slides and then labeled with 200µl of Qdot nanocrystal labeling solution. Slides containing cells that had been through the labeling process, with the exception of the Qdot reagent, were used as negative controls. The ratio of living to dead cells was assessed at 24 hours post labeling and again at 72 hours post labeling using a live/dead assay kit (LIVE/DEAD® Viability Cytotoxicity Assay Kit, Invitrogen/Molecular Probes). Images were captured using the Olympus digital light/fluorescence microscopy system, and the proportion of live and dead cells was counted. This was performed three times, a different cadaveric human donor cornea being used each time (n=3).

Labeling efficiency and duration of labeling in proliferating HLEC

HLEC colonies were established from 4 different cadaveric human donor corneas. Cells from each cornea were seeded into two wells of a 6 well plate, yielding 8 cultures in total. Once macroscopically visible colonies had formed, the cells were incubated with 1ml of Qdot labeling solution (as previously described) for 60 minutes. The cultures were washed three times with culture medium then incubated as normal. At 24 hours post labeling, images were collected using the Olympus digital light/fluorescence microscopy system. Ten images were collected from different HLEC colonies in each of 8 replicate wells. The number of Qdot containing cells was counted and this was expressed as a percentage of the total number of cells. The cultures were then passaged and reseeded onto fresh feeder layers at a density of 2000 cells per well of a 6 well plate. Six days later (day 7 following the initial labeling of cells), the percentage of cells containing Qdots was again evaluated. The cells were then passaged for a third and final time and the percentage of cells containing Qdots was counted again 14 days after the initial labeling.

Effect of Qdots on differentiation of HLEC

The differentiation state of HLEC was determined using immunofluorescence to assess expression of the putative LESC markers ABCG2 and p63alpha and the corneal epithelium differentiation marker CK3 (cytokeratin 3). HLEC were cultured in 4-well chamber slides

and labeled with Qdots. Slides containing unlabeled cells were used as negative controls. Five days after labeling, cells were washed, fixed with 3.2% paraformaldehyde (ABCG2/CK3) or methanol (p63alpha) for 5 minutes at room temperature, washed with PBS containing 0.5% triton X-100 (Sigma), and blocking of nonspecific primary antibody binding was performed using 10% goat serum in PBS for 30 minutes. Incubation with primary antibodies to ABCG2 (Chemicon), p63alpha (Cell Signalling Technologies) and CK3 (Santa Cruz Biotechnologies) was carried out overnight at 4°C. The slides were washed and incubation with the secondary antibody was performed at room temperature for 2 hours. Slides were then washed and mounted using Vectashield containing DAPI (Vector Labs). Slides were examined and images collected using a Zeiss LSM 510 confocal microscope.

Effect of Qdot labeling on colony forming efficiency of HLEC

HLEC were isolated and 200 cells were seeded into each well of a 6-well plate containing a mitomycin-C (MMC) growth arrested 3T3 feeder layer. On day 1 post seeding, cells in half of the wells were labeled with 1ml of Qdot nanocrystal labeling solution for 60 minutes followed by three washes with culture medium. The remaining unlabeled HLEC served as controls. HLEC were cultured until macroscopically visible colonies had grown. This took between 7 to 10 days following cell isolation. The presence of Qdots within colonies was evaluated by fluorescence microscopy. Cells were then fixed with methanol at -20°C for 30 minutes, rehydrated with PBS and stained with 1% Rhodamine B for 30 minutes at 37°C, washed with copious amounts of water and photographed. The number of colonies >2mm in diameter was counted and expressed as a proportion of cells seeded (colony forming efficiency).[16, 17] Clonal analysis was not performed.

Tracking of transplanted cells in an organ culture model

Human corneo-scleral rims were obtained from the Moorfields Lions Eye Bank. They were decellularised by incubation in 100mM EDTA (Sigma) for 2 hours at 37°C followed by gentle mechanical abrasion of the epithelium. This protocol was effective in removing virtually all the native epithelial cells as previously described (supplementary figure 9).[15] HLEC were isolated as above and cultured in a 12 well plate until they formed confluent epithelial sheets. Once confluent they were incubated with 500µl of Qdot nanocrystal labeling solution for 60 minutes then rinsed twice with culture medium. Dispase II (Roche Diagnostics Ltd, East Sussex, UK) was used to separate the intact sheet of cells from the culture plate. The cell sheet was transplanted onto the limbus of a decellularised corneal scleral rim and cultured for 2 weeks. At 7 days and 14 days post transplantation the sample was examined using a Zeiss LSM 510 confocal microscope.

Development of method of tracking Qdots ex-vivo using existing clinical technology

To determine whether existing confocal microscopes in clinical practice could be used to detect Qdots, the Rostock corneal module (Heidelberg Engineering) for the Heidelberg Retinal Tomograph II (HRT II) was modified to enable its attachment to the Heidelberg Spectralis (HRA II/OCT). This modification required the fabrication of a plastic sleeve that fitted around the imaging head of the HRA II that enabled the Rostock corneal module to be securely fitted onto it and the position of the Rostock module to be adjusted.

Fresh whole cadaveric rabbit eyes were obtained from an abattoir. Rabbit corneas were chosen because their size made handling easy and because a ready supply was available. The globes were sterilised by washing with 5% povidone iodine for 1 minute then rinsing 3 times in antibiotic and antimycotic solution (100 IU/ml each penicillin/streptomycin and 0.25 µg/ml amphotericin B, Gibco Life Technologies). The corneoscleral disc was removed from the globe and placed epithelium down in the well of a 6-well plate containing 3ml of Qdot nanocrystal labeling solution so that the entire corneal epithelium was submerged. The tissue was placed in a tissue culture incubator for 4 hours at 37 C. The corneas were then removed, washed twice with culture medium and incubated in moist chambers. At 12 hours post labeling the corneal epithelium was imaged using the Rostock corneal module attached to the HRA-II.

Results

Labeling of HLEC in culture

Intracellular Qdots demonstrated a broad excitation range between 405–615nm. In contrast they had narrow range of emission wavelengths with a peak at 655nm. The pattern and intensity of signal from labeled cells was very homogeneous. The appearance of these particles on fluorescence microscopy and the morphology of labeled cells HLEC are demonstrated in figure 1.

Confocal and electron microscopy confirmed the intracellular location of Qdots

Confocal and electron microscopy demonstrated that at 24 hours post labeling Qdots were present in membrane-less aggregates freely distributed throughout the cytoplasm of labeled cells (figure 2). At 1 week post labeling Qdots were located primarily in the nucleus but some cytoplasmic aggregates were present which on electron microscopy were found to be encapsulated by a membrane and associated with the Golgi apparatus. At 2 weeks post labeling Qdots were located primarily in the nucleus (figure 2).

Toxicity of Qdot in labeled HLEC

There were no differences in the number of living or dead cells between labeled and unlabeled cells at 24 hours or 72 hours post labeling (figure 3).

Labeling efficiency and duration of labeling in proliferating HLEC

The labeling efficiency at 24 hours post labeling was $94.6 \pm 3.3\%$ (mean $\pm$ SEM, n=8). Continued proliferation resulted in a gradual reduction in percentage of cells containing Qdots to $63.0 \pm 4.6\%$ at 7 days and $22.4 \pm 6.3\%$ at 14 days post labeling.

Effect of Qdots on colony forming efficiency and differentiation of HLEC

The colony forming efficiency of labeled HLEC ($8.5 \pm 1.8\%$) (mean $\pm$ SEM) was similar to that of non labeled HLEC ($10.0 \pm 2.9\%$) (figure 4). There was no significant difference between groups (t-test). This indicates that neither the labeling process, nor the presence of Qdots within cells interferes with the capacity of epithelial cells to form colonies in culture.

The degree of differentiation of HLEC was determined using immunofluorescence to assess the expression of the putative LESC “markers” ABCG2 and p63alpha and the corneal epithelium differentiation marker CK3 (cytokeratin 3) (figure 5). The pattern and distribution of expression of these ligands was identical between groups.

Tracking of transplanted cells in an organ culture model

Sheets of cultured HLEC labeled with Qdots were successfully transplanted onto decellularised human corneo-scleral rims in an organ culture model and observed for up to 14 days to investigate the behaviour of transplanted cells (figure 6). To ensure that the decellularisation protocol was successful in removing all of the native limbal epithelium, decellularised rims that did not receive transplants of cultured HLEC served as a control. These were cultured for up to 14 days alongside those rims that did receive transplants of labeled HLEC. No viable cells were found on the surface of the control sample at any time point indicating the effectiveness of the decellularisation process (data not shown).

Results of transplantation at 7 days—At 7 days post transplantation an intact epithelial sheet covered the entire cornea and limbus. A substantial proportion of these cells contained Qdots indicating that the transplanted cells had survived for the first 7 days. It was noticeable that in the limbal region, more cells still contained Qdots than those on the peripheral cornea. Also, the limbal area contained clusters of very Qdot bright cells. These were not present on the cornea (figure 7).

Results of transplantation at 14 days—At day 14 the number of Qdot labeled cells had decreased but there was still an intact layer of epithelium covering the corneal and limbus. The decline in Qdot signal was greatest in the cornea and less in the limbus. The clusters of Qdot bright cells were still present in the limbal region at 2 weeks, but they were less raised and prominent than initially (figure 7).

Development of method of tracking Qdots in vivo using existing clinical technology

Using the Rostock corneal module attached to the Heidelberg HRA-II it was possible to label and observe epithelial cells in rabbit corneas *ex-vivo*. The HRA-II contains three different wavelength lasers for excitation and a range of emission filters. The optimal configuration for detecting Qdots was found to be the same as that for performing fundus auto fluorescence scanning, i.e. the 488nm solid-state laser was used for excitation and emitted light detected above 500nm using a long pass filter. The appearance of Qdot labeled rabbit corneal epithelial cells is demonstrated in figure 8. Although fluorescence from Qdots could be detected with ease, the quality of images of individual rabbit epithelial cells was poor.

Discussion

The clinical use of *ex-vivo* expanded HLEC to treat patients with LSCD has preceded a clear scientific understanding of the mechanism of action of this therapy. Specifically, it is unclear whether *ex-vivo* cultured HLEC cell transplants re-integrate into the recipient’s LESC niche and continue to function in the long term to maintain a healthy corneal epithelium. An

alternative hypothesis is that the temporary restoration of a healthy corneal epithelium by the transplanted cells acts as a biological bandage and promotes regeneration of the recipient's own LESC population. This question could most definitively be answered by tracking transplanted cells *in-vivo*. This has not been performed to date, not least due to the lack of laboratory data on labeling efficiency, duration, toxicity and effects on transplanted HLEC function. The aim of this study was to determine whether Qdots could be used to label and track *ex-vivo* cultured limbal epithelial cells without impacting upon cell viability and function. We have shown that Qdots have potential in this regard and have demonstrated proof of principle for their use in tracking transplanted cells in the cornea. Lastly we demonstrated how existing clinical equipment could be modified to enable *in-vivo* detection of Qdot labeled cells in animal or human studies.

The ability to follow the distribution and migration of biologically active cells in living organisms is crucial for both the development of cell-based therapies and for the elucidation of biological mechanisms in stem cell research. Two key components are required to achieve this: A method of selectively labeling transplanted cells and a technique for detecting and visualising the transplanted cells *in-vivo*.

There are two distinct approaches to cell labeling: indirect and direct labeling of cells. Indirect labeling methods use a reporter gene that is introduced into a cell and translated into enzymes, receptors, or fluorescent or bioluminescent proteins. If the expression of the reporter gene is stable, labeled cells can be observed over the entire lifetime of cells. Indirect labeling has proved an immensely valuable tool in terms of basic research but there are significant obstacles to the translation of this technology into clinical practice, not least the ethical and regulatory issues that result from the use of genetically modified cells. Direct cell labeling refers to attachment of exogenous detectable labels (fluorophores, magnetic particles or radio-isotopes) to the cell membrane or their intracellular incorporation using a variety of techniques. Direct labeling is technically straightforward and does not involve genetic modification of the cell. It is therefore a safer and more clinically useful approach and the one we chose to employ.

By virtue of its transparency, the cornea is an ideal tissue in which to directly observe transplanted stem cells using a fluorophore label and *in-vivo* microscopy. This combination can achieve a level of resolution that other detection systems such as MRI, SPECT and PET cannot.[10] When selecting a fluorophore we set the criteria that it should be non-toxic, photostable (i.e. resistant to photobleaching) and sufficiently bright to generate a signal detectable through living tissues. We chose to use Qdots because they exhibit some important differences as compared to traditional fluorophores such as organic fluorescent dyes and naturally fluorescent proteins.

Structurally, Qdots are inorganic composites consisting of nanometer-scale (roughly protein-sized) clusters of atoms (containing from a few hundred to a few thousand atoms) of a semiconductor material (cadmium mixed with selenium or tellurium been the most common for biological applications), which has been coated with an additional semiconductor shell (ZnS) to improve the stability and optical properties of the material. Qdots fluoresce in a completely different way than do traditional fluorophores. In Qdots fluorescence is achieved

via the formation of excitons, or Coulomb correlated electron-hole pairs that are created between the two semiconductor materials. The exciton can be thought of as analogous to the excited state of traditional fluorophores; however, excitons typically have much longer lifetimes (> 10 ns, up to ~ 200 nanoseconds, vs. < 5 ns for organic fluorophores).[18] This structural and functional difference engenders two key advantages on Qdots over conventional fluorophores. Firstly, photostability of Qdot nanocrystals is many orders of magnitude greater than that associated with traditional fluorescent molecules and hence photo-induced deterioration of the emitted signal (known as bleaching) is substantially less than that seen with other types of fluorophores. Secondly, the optical efficiency of Qdots (quantum yield, in general terms the amount of light emitted relative to that absorbed) is many times that observed for other classes of fluorophores. As a result less illumination is required to achieve a detectable signal from Qdots than from other classes of fluorophores. One more general advantage over classic fluorophores comes from multiplexing. Different colour Qdots can be excited with a single wavelength and the narrow emission band of Qdots makes deconvolution straightforward.

The commercial Qdot kit we used employs a custom cell penetrating peptide bound to the surface of Qdots to induce their cellular uptake. [19, 20] These peptides are a beta-amino acid analogue of Tat 47-57, the portion of the HIV Tat DNA-binding protein that is responsible for the ability of the HIV Tat protein to readily cross the cell membrane.[21] Tat 47-75 analogues have negligible cytotoxicity in HeLa, Chinese Hamster Ovary (CHO-K1), and Human Umbilical Vein Endothelial Cells.[22] The corneal toxicity of cell penetrating peptides has been assessed in a rabbit drug delivery model and found to be negligible.[23] The intracellular target of Tat 47-57 and its analogues is the nucleus.[19, 20] In the initial 24 hours after labeling Qdots formed membrane-less aggregates throughout the cytoplasm, presumably reflecting the fact that they are trafficking to the nucleus. By 1 week post labeling Qdots were primarily located in the nucleus as expected. Some cytoplasmic aggregates were still present at this time point and on electron microscopy these were found to be encapsulated by a membrane and associated with the Golgi apparatus. These findings suggest that some metabolism of and possibly exocytosis of Qdots were occurring at this stage. By 2 weeks post labeling Qdots were located primarily in the nucleus with few cytoplasmic aggregates suggesting that by this time point the labeling was stable. There is evidence that mouse embryonic stem cells can both degrade and exocytose the same commercial Qdot system that we have used resulting in loss of Qdot signal within 48 hours of labeling.[24] In contrast the same study found that mouse embryonic fibroblasts did not degrade or exocytose Qdots and that the reduction in signal seen over time in this cell type was due to the diluting effect of cell division. Our results show that Qdots can result in a labeling efficiency of over 94% immediately post labeling which gradually reduced to 22% after 14 days as the cells proliferated. This is remarkably similar to the findings in mouse embryonic fibroblasts [24] and we therefore hypothesize that whilst limited metabolism and exocytosis do occur in HLEC, the primary reason for the reduction in Qdot signal over time is dilution due to cell division.

Another significant limitation of our organ culture model is that we have been unsuccessful in maintaining limbal epithelial cell sheets (both labeled and unlabeled) in culture on stromal

explants for longer than 2 weeks. This is because the transplanted epithelium differentiates rapidly between day 14 and 21 post transplantation. Although our model does not permit us to track cells beyond 2 weeks, this does not mean that quantum dot labeled cells will be undetectable beyond this time point. In-vivo studies will be necessary to determine the duration of labeling with certainty. Two-photon imaging has been used to demonstrate that Qdots can be retained within the cornea for up to 26 days in an in vivo mouse model albeit this was a model of toxicity and not a model of cell tracking.[25]

In order for Qdots to be used clinically it is essential for their toxicity to be investigated. It was encouraging to find that a high level of labeling efficiency could be achieved and that neither the labeling process nor the on-going presence of Qdots within cells caused toxicity or promoted differentiation. This is in contrast with one previous study by Kuok et al in which two-photon microscopic imaging was used to study the distribution of chemically similar (CdSe/ZnS core/shell) Qdots throughout the cornea. [25] Kuo et al found evidence of cytotoxicity in bovine corneal stromal cells. Cell viability decreased significantly as the Qdot concentration and incubation period increased. The reasons for the discrepancy between this study and our results could be that different cell types or even different species handle Qdots differently. Alternatively the different concentrations of Qdots used (50nM vs. 10µg/mL in our study) and exposure periods (24-48 h vs. 60 minutes in our study) reflect the fact that Kuo et al were seeking to define limits of toxicity whereas we were using an optimised and validated protocol for cell labeling.

When assessing the effect of Qdots on proliferation and differentiation of HLEC we did not perform clonal analysis. We used a mixed population cells which will have included holoclones, meroclones and possibly some paraclones. We cannot therefore be certain whether these results apply specifically to LESC (holoclones), transient amplifying cells (meroclones) or both.[16, 17]

Our organ culture transplantation model showed that we could still detect transplanted cells at 14 days post transplant. At present there is no reliable method of directly visualising transplanted ex-vivo cultured HLEC transplanted in human patients. Our techniques could be employed to monitor cells for up to two weeks and perhaps longer, depending on the rate of proliferation of transplanted cells. Whilst it would be desirable to be able to track cells for their entire lifetime, the technology to do this safely in-vivo is not currently available. The ability to track cells even for one month post transplantation would lead to significant advances in understanding the biological mechanism of action and post treatment behaviour of such transplants.

Based on the observations that LESC divide infrequently in-vivo and therefore retain BRDU longer than their differentiated progeny[26] and that the rate of proliferation determines the rate of label dilution and therefore duration of labeling, we hypothesise that post transplantation cells that divide rapidly, such as transient amplifying cells, will see the number of Qdots in their cytoplasm rapidly reduce to undetectable levels whereas cells that divide infrequently, as stem cells do, may retain a high number of Qdots. In our organ culture model of HLEC transplantation, it was observed that there was a marked heterogeneity of Qdot signal with the brightest cells containing most Qdots being found in

clusters at the limbus. This suggests that the limbus has an effect on the transplanted cells, which the cornea does not. It also suggests that some of these slow cycling cells may re-integrate into the limbus and remain in a slow-cycling or quiescent state. From our data it is not possible to confirm that these cells possess a limbal stem cell phenotype but further work will aim to investigate whether this is indeed the case.

In-vivo confocal microscopy has been increasingly used to investigate the cornea in health and disease. In particular the Rostock Corneal Module for the Heidelberg Retinal Tomograph (HRT) II has enabled cellular level investigation of corneal structure and the limbal stem cell niche.[15, 27, 28] Our results from ex-vivo rabbit corneas demonstrate that the Rostock Corneal Module can be modified to work with the Heidelberg Spectralis HRA-OCT to enable in-vivo detection of cells labeled with Qdots in-vivo. There is undoubtedly room for a more precisely engineered method of combining in-vivo confocal microscopy with fluorescence detection but the data we present demonstrates that this is technically possible.

Conclusions

This work demonstrates that Qdots can be used to label HLEC for up to 2 weeks in culture without causing toxicity or differentiation and that the technology exists to image Qdots in-vivo in humans. However, there are a number of obstacles that need to be overcome before Qdots can realistically be used clinically. The main limitation of this study is that our organ culture transplantation model only permits tracking of cells for 2 weeks at present. It is possible that higher resolution imaging, prevention of Qdot aggregation within cells and modifications to the designs of the Qdots themselves may prolong the duration of labeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support: This research was funded by a research training fellowship for AS from the Medical Research Council, UK and by research grants from Fight for Sight UK and the National Institute for Health Research (NIHR) Biomedical Research Centre based at Moorfields Eye Hospital NHS Foundation Trust and UCL Institute of Ophthalmology. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health."

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Figure 1.

Quantum dot labeling of primary human limbal epithelial cells.

Phase contrast microscopy (A&C) and fluorescence microscopy (B&D) of Qdot labeled primary human limbal epithelial cells using x 20 objective (A&B), and x40 objective (C&D). These images demonstrate that Qdots are not visible with phase contrast microscopy using white light. Images C&D demonstrate the corresponding area in A&C when viewed with a green wavelength light (450 – 550nm) and viewed using a red filter. The Qdots (red) are seen to fluoresce brightly and appear to be aggregated in clumps within individual cells.

Figure 2.

Intra-cellular localisation of Qdots.

Confocal microscopy (A – merged phase and fluorescence, B - fluorescence image) and electron microscopy (C & D) of Qdot labeled primary human limbal epithelial cells (HLEC) taken 24 hours post labeling showing the widespread distribution of Qdot aggregates throughout the cytoplasm. The cytoplasmic aggregates are not encapsulated by a cellular membrane but rather are free distributed. At 1 week post labeling confocal microscopy (E - merged phase and fluorescence image, F – fluorescence image) and electron microscopy (F & G) show that Qdots are primarily located in the nucleus. Some cytoplasmic aggregates are present at this time point, which on electron microscopy were found to be encapsulated by a membrane and associated with the Golgi apparatus (F). At 2 weeks post labeling Qdots were located primarily in the nucleus with few cytoplasmic aggregates (H – merged phase and fluorescence image & I - fluorescence image).

Figure 3.

Results of live dead assay to evaluate the toxicity of Qdots.

Unlabeled control cells (A) and cells labeled with Qdots (B) demonstrate a similar ratio of live to dead cells, scale bar 50µm. C) Quantification of the number of live (green bars) and dead cells (red bars) in both groups reveals no difference in the percentage of live or dead cells in either group. Error bars represent the standard error of the mean (n=3).

Figure 4.

Effect of Qdot labeling of primary human limbal epithelial cells (HLEC) on colony forming efficiency (CFE).

A) Photograph of CFE assay for HLEC labeled with Qdots demonstrating the presence of numerous colonies with classic holoclone morphology. B) Corresponding photograph for non-labeled HLEC demonstrating a similar number and size of holoclone colonies. n=3.

Figure 5.
Effect of Qdots on differentiation of human limbal epithelial cells (HLEC).
Confocal microscopic images of Immunofluorescence for the differentiation marker CK3 and putative stem cell “markers” p63alpha and ABCG2 at day 5 post labeling of HLEC.
Scale bar = 50µm.

Figure 6.

Method of culture and transplantation of a labeled sheet of human limbal epithelial cells (HLEC) in an organ culture model.

A) Fluorescence microscopy image of sheet of human limbal HLEC that has been labeled with Qdot (scale bar 50µm). B) Sheet of Qdot labeled HLEC that has been separated from the culture dish using dispase. C) Decellularised human corneoscleral rims in an organ culture chamber. D) Image obtained from recombined corneoscleral rim and HLEC sheet 7 days post transplantation using a Zeiss LSM 510 confocal microscope (scale bar 10µm).

Figure 7.

Results of transplanting labeled human limbal epithelial cells sheets onto decellularized corneoscleral rims.

Columns A and D indicate the location from which the adjacent confocal microscopic images were collected. Columns B and C demonstrated the presence of labeled HLEC on the surface of the cornea at 7 days and 14 days post transplantation. Columns E and F demonstrate the appearance of labeled HLEC on the surface of the limbus at 7 days and 14 days post transplantation. At 7 days post transplantation an intact epithelial sheet covered the entire cornea (B) and limbus (E). A substantial proportion of these cells contained Qdots indicating that the transplanted cells had survived for the first 7 days. It was noticeable that in the limbal region, more cells still contained Qdots (E) than those on the peripheral cornea (B). Also, the limbal area contained clusters of very Qdot bright cells (E). These were not present on the cornea (B). At day 14 the number of Qdot labeled cells had decreased but there was still an intact layer of epithelium covering the cornea (C) and limbus (F). The decline in Qdot signal was greatest in the cornea (C) and less in the limbus (F). The clusters of Qdot bright cells were still present in the limbal region at 2 weeks, but they were less raised and prominent than initially (F).

Figure 8.

Ex-vivo confocal microscopy of rabbit corneal epithelium labeled with Qdots

Images were acquired using the Rostock corneal (Heidelberg) module modified to attach onto the Spectralis OCT. clinical imaging equipment. A&B unlabeled control rabbit cornea. Individual corneal epithelial cells can be distinguished. C&D Qdot labeled rabbit corneal epithelium. The white dots scattered throughout the field of view are the Qdot aggregates within the cytoplasm of labeled cells. Scale bar 50 μ m.