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CdSe quantum dots evaluation in primary cellular models or tissues derived from patients☆

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Abstract

In recent years quantum dots (QDs) have risen as useful luminescent nanoparticles with multiple applications ranging from laser, image displays and biomedical applications. Here we review and discuss the studies of these nanoparticles in patient derived cellular samples or tissues, including cellular models from iPSCs from patients, biopsied and post-mortem tissue. QD-based multiplexed imaging has been proved to overcome most of the major drawbacks of conventional techniques, exhibiting higher sensitivity, reliability, accuracy and simultaneous labeling of key biomarkers. In this sense, QDs are very promising tools to be further used in clinical applications including diagnosis and therapy approaches. Analyzing the possibilities of these materials in these biological samples gives an overview of the future applications of the nanoparticles in models closer to patients and their specific disease.

Key words: CdSe quantum dots; Human tissue; Primary human cells; Diagnostics

One of the most challenging tasks in medical sciences is clinical translation. In order to overcome this problem, there is a trending line of research that aims at working closer to human derived cells, tissues or post-mortem samples that utilize specific samples from patients to understand and treat diseases rather on relying on models derived from cell lines. While human commercially available cell lines have represented a unique source of cellular models to study diseases, they barely represent the nature of the target cells they are intended to represent.[1] They are cultures that come in origin from a limited sample of material, which have gone through multiple passages and behave in a clonal manner and therefore, there is a movement to use cells derived from patients such as iPSC, peripheral circulating cells or cells obtained from biopsies, which are an exceptional source of material.[2]

Quantum dots (QDs) are semiconductor nanoparticles formed by a CdSe core and ZnS coating that offer unique photo luminescent properties. They present an enhanced photostability, large molar extinction coefficients, broad absorption and narrow emission spectra and size-dependent color tunability. These improved properties enable higher detection sensitivity, decreased photobleaching and the possibility of efficient multiplexed analysis compared to organic dyes.[3] The surface of the QDs has been designed with coatings with hydrophilic ligands such as poly (ethylene glycol) (PEG) or amphiphilic polymers encapsulation, in order to make QDs water soluble and appropriate for biological applications.[4] Advances in coating development and bioconjugation techniques on the surface of QDs allow the conjugation of different biomolecules of interest, representing high adaptable tools for targeting distinct molecular entities. In this sense, QDs are very promising tools to detect molecular mechanisms at the subcellular level, allowing the characterization of physiologic and pathologic mechanisms underlying disease processes.[5]

While the use of QDs in cell line cultures,[5] animal specimens,[6,7] and human fluids has been abundant,[8,9] the use of these nanoparticles to characterize human primary cells or tissue directly derived from patients has been much more limited, which hampers an efficient translation of these chemical tools to the clinic. Several studies show how QDs have been successfully

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employed in studies involving primary cells or tissue derived with patients with different purposes, showing higher sensitivity and photostability than conventional techniques and providing new insights in clinical diagnosis.[10–12] In this review we aim to give a general overview of the methodologies employed with QDs in human derived cells and tissues and post-mortem samples, and highlight their advantages over other traditional techniques.

Commercially available quantum dot tools

Currently, a wide-number of QD probes coupled to proteins, antibodies or peptides are commercially available (Figure 1). Among them, the most used QD bioconjugates are coated either with streptavidin (for conjugation with biotinylated antibodies or proteins) or with antibodies (mainly specie-specific IgG/secondary antibodies). The commercial availability of different QD bioconjugates has increased their use as biological tools for different applications including diagnostic purposes. Unlike conventional techniques for tissue staining such as hematoxylin and eosin (H&E) and immunohistochemistry (IHC), these multicolor QD bioconjugates can simultaneously detect a panel up to four or five protein biomarkers directly on human tissue specimens.[13,14] However, QD conjugates also present some disadvantages. The use of QD conjugated to streptavidin (QD-SA) is limited by the availability of biotinylated primary antibodies that label the desired target. If no biotinylated primary antibodies for a specific target are available, a 3-step immunofluorescence, primary antibody, secondary biotinylated antibody, and QD-SA, can be performed. In fact, this strategy will allow multiplexed labeling using QD-SA. Nevertheless, as well as QD conjugated to secondary antibodies (QD-Ab2), their use is also limited by the availability of primary antibodies from different animal species (mouse, rabbit, rat…).

In this sense, while the most straightforward option to perform experiments with QD conjugates is to directly purchase the probe needed, it is still not yet a reality. Usually the user needs to adapt their experiments to these QDs conjugates or to modify QD coating to obtain the tool required. In order to customize QD surface, multiple techniques have been developed so far, including hydrazine ligation, strain-promoted azide-alkyne cycloaddition, amide coupling and polyhistidine assembly.[3]

Additionally, one of the major drawbacks that prevent QDs to be used regularly in research centers and clinical diagnostic facilities may be the lack of homogeneity and reproducibility between commercially available QDs batches. Although different functionalized QDs are currently accessible, precise specifications about their characterization, size, number of functionalized groups per QD and other key characteristics of the nanomaterials are not clearly provided by the provider, limiting their potential use.[15–17] In an attempt to offer consistent and reproducible methods to conjugate different biomolecules of interest to QDs, commercial labeling and conjugation kits have been developed, although the ratio and orientation of biomolecules conjugated are difficult to control and confirm. Also their high price prevents more spread of utilization of these novel materials.

Another important factor to be considered is QD toxicity in in vitro models. There have been multiple studies that have extensively analyzed this topic, coming to the conclusion that the toxicity depends on the physicochemical properties, coating and stability of each QD.[18–22]

Despite these challenges, QD imaging provides an amount of benefits including better sensitivity and more consistent linear relationships for biomarker quantification than conventional techniques such as IHC or H&E.[23]

QDs enhanced photoluminiscent properties allow to overcome detection limits in immunostaining samples and to serve as high sensitivity and reproducibility tools. Their multiplexed capability has opened a gateway for screening for a large panel of biomarkers to uniquely identify molecular fingerprints of the disease.[24] It also enables the colocalization of many targets within the same sample.[15] Furthermore, their practical use in simple devices promises their future utilization at point-of-care centers, bringing diagnostic tools based on nanomaterials closer to the patients.[25,26]

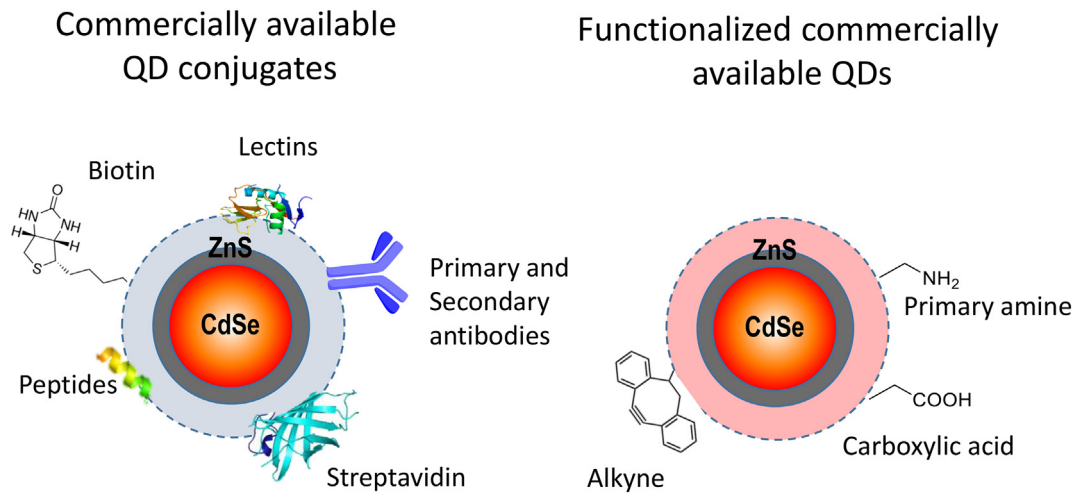

Figure 1. Commercially available QD conjugates for biological experiments. (**A**) Ready to use QD conjugates; (**B**) functionalized QDs ready to attach the desired biomolecule of interest.

Multiple studies, summarized in this article, have showed the utility of QDs for clinically relevant applications, including diagnosis, toxicity and cellular tracking in human primary samples derived from patients. The combination of two state of the art techniques, QDs and human primary cells or tissue specimens, provides new insights that could have not been addressed with conventional immunofluorescent techniques or commercially available cell lines.[18,27] In this review we analyze the utilization of these materials on these biological samples in order to give an overview of their advantages and promises. The review includes results on disease diagnosis and characterization, QD nanosafety and cellular tracking.

Disease diagnosis and characterization

IHC and H&E are the most common techniques used for diagnostic and characterization purposes. IHC is a widely established method for studying molecular pathology biomarkers in tissue specimens for diagnostic purposes and clinical research.[28,29] It is a high sensitivity and low cost technique that can provide both geometrical and functional information about biological samples.[30] However, simultaneous imaging of different targets is technically challenging for IHC due to poor specificity and low reproducibility. Therefore, imaging of multiple antigens is not available using IHC. Regarding H&E staining, it has also some limitations, for example in cancer, even though it exhibits the histological structures of the tumor, it does not always enable the differentiation between the tumor and the stroma.[31] Organic dyes have also been employed to perform fluorescent IHC staining of two or more antigens. However, organic dyes present also some disadvantages like low signal intensity, photobleaching and spectral overlapping.[14]

QDs have made a remarkable impact in imaging techniques, including diagnosis of pathological conditions, with oncology being one of the fields in which they have been abundantly utilized.[32] Cancer diagnosis is a fundamental field of research that has helped to better understand the pathology, appropriately classify the patients and overall improve cancer prognosis increasing patient survival in the last years. The most important and challenging difficulties in studying the mechanism of cancer are tumor heterogeneity and complexity.[33] Thus this level of complexity requires tools that can provide results at the single-cell level avoiding data obtained by ensemble averaging over heterogeneous cell populations that mask individual responses and behaviors. QDs properties make them extremely useful in multiplexed molecular imaging to identify molecular fingerprints of diseases, which is the main goal of personalized medicine. In particular, the multiplexed capability of QDs is well-suited for studying the pathological molecular characteristics of different tumor subtypes.[34] They have been used to study the molecular pathology signatures of different subtypes of tumors by a multi-biomarker screening via multiplexed detection on patients' samples.[35]

The studies are classified in this review depending on the type of QD employed in the experiment, commercially QD conjugates with secondary antibodies, QD-streptavidin conjugates and QD covalently linked to antibodies or other biomolecules of interest such as peptides or ligands. (Table 1)

Finally, we remark some safety, uptake and distribution studies that have been performed in primary tissues, bringing new insights of the behavior of QDs in these systems.

QD conjugates with secondary antibodies

Commercially available QD conjugates with secondary antibodies have opened the possibility of direct employment of QD probes without the need of further functionalization. In a recent work, Chen and colleagues performed a QD-based multiplexed imaging with these probes in heterogeneous circulating tumor cells (CTCs) captured from patient's blood samples. They performed a comparison of staining methods in which no statistical significance difference was observed in the detection ability between QD staining and organic dyes, validating QD results. Interestingly, QDs exhibited stronger photostability, allowing for the simultaneous imaging of mesenchymal and epithelial CTCs.[36]

Similarly, QD-secondary antibody conjugates (QD-Ab2) were used by Li′s laboratory with the objective of gaining new insights into the mechanisms of tumor invasion that could lead to develop new anti-cancer therapies. The interaction between cancer cells and stroma together with the spatiotemporal process of tumor invasion was studied in 10 breast cancer (BC) and 15 gastric cancer (GC) patients. A 3-multiplex QDs staining method was used to recognize major components of tumor microenvironment involved in cancer invasion processes such as extracellular matrix (ECM) remodeling (IV type collagen and matrix metalloproteinase 9, MMP9), tumor angiogenesis (CD105, endothelial cells) and immune cell infiltration (macrophages). Four different patterns of tumor invasion, washing pattern, amoeba-like pattern, polarity pattern and linear pattern with different co-evolution features were identified, enhancing the development of preventative and therapeutic strategies that target specifically the microenvironment found.[11] Moreover, the QDs-based multiplexed technology was also useful for assessing the prognostic value of tumor stromal features in GC. The spatial and temporal distribution of tumor stromal characteristics including infiltrating macrophages, neovessels, and type IV collagen, was simultaneously and quantitatively studied using QDs since it could not be performed using conventional techniques.[37] In a later study, the 3-multiplex QDs staining was combined with clinical research, allowing the authors to develop a cancer invasion mode called “pulse-mode” to accurately comprehend the evolution of tumor microenvironment and cancer cells during cancer progression.[38] In another line of research, Li and co-workers also studied the dynamic changes of ECM degradation during BC invasion. A QD-based double imaging of Her2 on BC cells and type IV collagen in the ECM was performed in human BC specimens from patients with different Her2 status (no Her2 expression, Her2+, Her2 2+, Her2 3+). Her2 overexpression is associated with a greater destruction of the ECM. Indeed, an increase of Her2 and an excessive degradation of type IV collagen could be used in BC diagnosis to assess the degree of malignancy and the invasive potential. Conventional single marker IHC staining of Her2 and type IV

Table 1
Summary of results obtained with QDs in primary human cells and tissues and the technical advantages observed.

Reference	QD type	Primary cells or tissue type	Number of patients	QD technical advantages	Results found
[42]	QD with secondary antibodies	Heterogeneous circulating cells	55 cancer patients: breast (12), lung (13), gastric (15) and colorectal (15) and 8 healthy donors	Stronger photostability, simultaneous imaging, similar staining pattern to organic dyes	Development of a reliable method for capture and detection of circulating tumor cells.
[11,37,38]	QD with secondary antibodies	Gastric cancer tissue and breast cancer tissue	1st study with 15 gastric cancer and 10 breast cancer patients. 2nd study with 184 gastric cancer patients	QD multiplexed mapping	Four patterns of tumor invasion identified. Prognosis value in gastric cancer with the evolution of tumor microenvironment
[39–40]	QD with secondary antibodies	Brest cancer tissue	33 breast cancer patients	Multiplex staining. Signal separation and quantification straightforward	Diagnosis of malignancy and invasive potential of BC tumors. Prognostic value in BC specimens
[14]	QD with secondary antibodies	Prostate cancer tissues	16 prostate cancer patients	High throughput digital mapping method	Single malignant tumor cells identification, applications in clinical diagnosis
[10]	QD with secondary antibodies	Hodgkin's lymphoma tissue	6 Hodgkin's lymphoma patients, 2 suspicious cases, and 2 patients with reactive lymph nodes	Multiplex staining and cleaner background	QD staining pattern accurately detected Hodgkin lymphoma
[43]	Streptavidin QD conjugates and directly labeled QDs	Human tonsil and lymphoid tissue	Tonsil and lymphoid tissues	Higher brightness and lower background than QD-AB2, multiplex	Staining of 5 common lymphoid biomarkers
[46]	Streptavidin QD conjugates	BC tissue	HER2 positive tissues	Higher fluorescence intensity and photostability than FITC	Labeling of tumor marker Her2
[47–48]	Streptavidin QD conjugates	BC tissue	700 patients, 94 clinical samples of BC	More sensitive, accurate and economic than IHC	Discovery of novel BC subtypes with differential prognosis
[50-51]	Streptavidin QD conjugates	BC tissue	Complete tumor specimens from 240 invasive BC patients, 75 clinical specimens	Significantly higher positive rates than IHC. Possibility of simultaneous staining	New insights in cancer specimens heterogeneity
[52]	Streptavidin QD conjugates	BC tissue	240 breast cancer patients	Better correlation and reliability than IHC	Prognostic value of EGFR in Her2 + patients
[56]	Streptavidin QD conjugates	BC tissue	108 breast cancer patients	Easy, accurate and sensitive method to evaluate Ki67 expression. Better interobserver agreement than IHC	Prognostic value of Ki67 in Her2+ patients
[31]	Streptavidin QD conjugates	BC tissue	240 Breast cancer patients	Multiplex staining	Ki67/CK as new parameter to better predict BC prognosis
[57]	Streptavidin QD conjugates	Lung cancer tissue	70 cases of lung carcinomas	Excellence photostability, improved signal to background ratio	Advantageous labeling of caveolin-1 and proliferating cell nuclear antigen
[58]	Streptavidin QD conjugates	BC tissue	58 breast cancer patients and 57 patients with benign breast pathology	Higher specificity and sensitivity achieved in Tn antigen labeling compared to HRP staining	Quantitative specific Tn antigen labeling
[59,60]	Streptavidin QD conjugates	Prostate cancer tissue	44 prostate cancer patients	Higher specificity and intensity than QD-Ab2. Detection of 4 biomarkers, sample variability reduction	Prediction of the survival and progression of PC patients
[61]	Streptavidin QD conjugates	Prostate cancer tissue	54 primary prostate cancer specimens	Multispectral imaging	Discovery of survival prediction patterns in different patients
[62]	Streptavidin	Prostate cancer	Prostate cancer tissue	Optimized multiplex in situ	miRNAs correlation with bone

Table 1 (*continued*)

Reference	QD type	Primary cells or tissue type	Number of patients	QD technical advantages	Results found
	QD conjugates	tissue		hybridization staining	metastases and patient survival
[63]	Streptavidin QD conjugates	Prostate cancer tissue	Tissue microarray with 840 radical prostatectomy cases	Better reproducibility than IHC. Computerized quantitative assessment of Akt	High levels of Akt1 indicate biochemical recurrence and death. Discovery of a novel prognostic biomarker
[64]	Streptavidin QD conjugates	Lung colon prostate breast liver and kidney cancer tissues	Human cancer tissue	Effective and specific labeling. Double QD labeling	Insights in the potential of near infrared dyes for the detection of cancer
[65]	Streptavidin QD conjugates and QD with secondary antibodies	GC tissue	118 gastric cancer patients	Enabling an holistic approach to study heterogeneity in cancer specimens	Discovery of LC3B correlation with lower invasion value and predictive value of death by Cav-1
[66]	Streptavidin QD conjugates	Lynch syndrome tumor sections	36 patients and 6 healthy donors	Advantage in simultaneous marker staining, however lower utility in the identification of mutation carriers	Worse performance in the identification of patients with mutations than IHC
[12]	Covalent conjugates with primary Ab	Primary cells from BC biopsies	Primary culture from breast cancer tissue	Quantitative multiplex cytometry with higher sensitivity	Accurate identification of tumor heterogeneity that allows an improved patient classification
[13]	Covalent conjugates with primary Ab	BC tissue	Clinical tissue specimens	Multiplex staining. Excellent correlation with IHC	Molecular profiling in cancer cells with potential clinical application
[67]	Covalent conjugates with primary Ab	Renal cancer tissue	25 tissue specimens of renal cell carcinoma and 3 healthy specimens	Simultaneous labeling of different cancer biomarkers	Accurate diagnosis for renal cell carcinoma in patient's tissue
[69]	Covalent conjugates with primary Ab and commercial QD-Ab2	Head and neck cancer tissue	Head and neck cancer tissue	High photostability	High intensity of fluorescence after 5 months
[70]	Covalent conjugates with primary Ab	*Plasmodium falciparum* infected erythrocytes	Human erythrocytes	Higher stability and lower photobleaching than organic fluorophores	Alterations of the membrane during parasite infection were found. Efficient labeling of band 3 protein
[71]	Exosomes from cancer stem cells	QD-cytokine conjugate (IL13)	Human glioma stem cells	Specific targeting of tumor associated exosomes	Validation of IL13QD as an ex vivo marker in diagnosis and prognosis of glioma

collagen was also performed finding a good correlation between QD and IHC images, validating these results.[39] The co-expression and the individual impacts of Her2 and Ki67, both key biomarkers in BC, have been studied in tissue specimens from 33 invasive BC patients using a QD-based double imaging. Results demonstrate that QDs made signal separation and quantification straightforward due to their enhanced photoluminiscent properties. This study allowed authors to prove that both biomarkers' expression significantly correlates with 8-year disease free survival (DFS). Also, due to the multiplexing capability of QDs, they established that Ki67 has a higher negative effect on BC prognosis than Her2 since the median 8-DFS was significantly shorter in high-Ki67 high-Her2 patients (11.7 moths) than in low-Ki67 high-Her2 patients (60.1 months).[40]

Multiplexed imaging of several key targets has allowed in all studies a higher degree of sensitivity and selectivity in cancer imaging that could not have been achieved using traditional

H&E and IHC, enabling the unraveling of the mechanisms underlying tumor invasion. Additionally, these experiments allow to take the most out of a limited and valuable material obtained directly from patients.

A high-throughput digital mapping method based on QD-Ab2 (Figure 2) was developed by Liu and colleagues and implemented in a cellular model derived from prostate cancer patients.[14] 4 protein biomarkers (E-cadherin, high-molecular-weight cytokeratin, p63 and α-methylacyl CoA racemase) were labeled simultaneously on human prostate cancer tissues. QD-Ab2 conjugates could successfully identify single malignant tumor cells and provide new molecular and morphological features from mapping the molecular, cellular and glandular heterogeneity of clinical tissue specimens from 16 prostate cancer patients (Figure 3). In contrast with conventional techniques, multiplexed QD mapping opened up correlated glandular architecture and molecular biomarkers information for clinical diagnosis applications. This QD multiplexing approach was also applied to the characterization and the identification of low abundant cancer cells[41] Hodgkin Reed-Sternberg (H/RS) in Hodgkin's lymphoma, a task that is critical for its diagnosis.[42] Conventional techniques were used to detect H/RS cells but the noisy cellular background hampered decisive diagnosis, due to the incapability of multiplexing. A panel of four biomarkers (CD15, CD30, CD45 and Pax5) was directly labeled in human tissue specimens from 6 patients with confirmed Hodgkin's lymphoma, 2 ambiguous lymphoma cases and 2 patients that have reactive lymph nodes (but not lymphoma). Results provided a distinct QD staining pattern (CD15 positive, CD30 positive, CD45 negative, and Pax5 positive) to detect H/RS cells

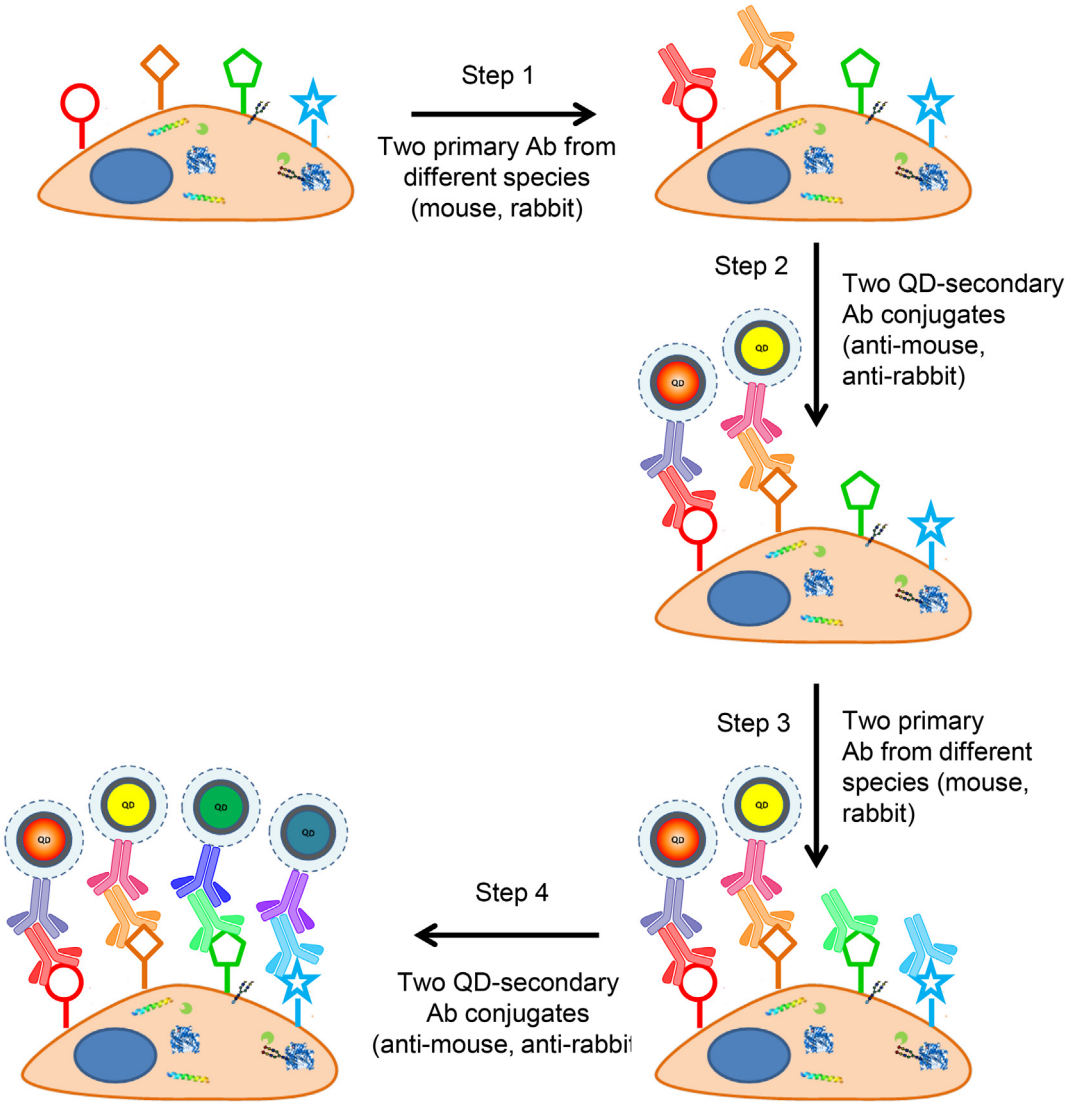

Figure 2. Sequential QD based immunofluorescence used in primary cells derived from prostate cancer patients. Two primary antibodies from different animal species are used to recognize two different antigens. (step 1). Then, two QD-Ab2 are applied to stain the two primary antibodies (step 2). After washing, the same procedure is repeated (step 3 and 4) to finally simultaneously stain up to 4 different antigens. *Adapted with permission from Liu et al.*[14] *Copyright 2010 American Chemical Society.*

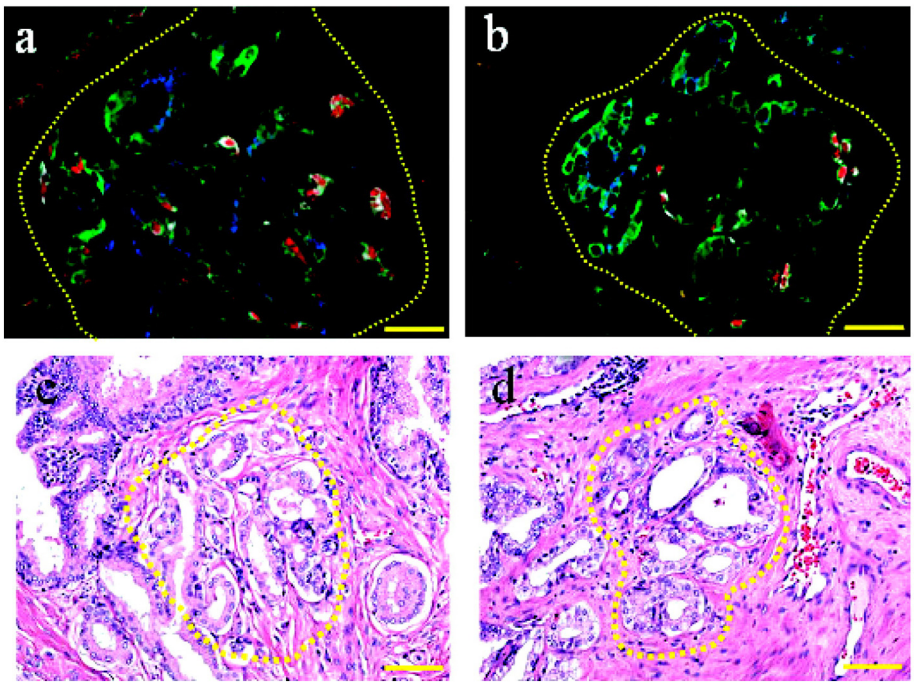

Figure 3. Comparison of multiplexed QD mapping (**A, B**) and traditional H&E. (**A**, **B**) Multiplexing QD imaging and (**C**, **D**) IHC for two histopathologically complex foci on adjacent prostate cancer tissue sections. The dashed circles in images **A** and **C** correspond to one complex region, and the dashed circles in images **B** and **D** correspond to another complex region. In panels **A** and **B**, objective 40, scale bar 50 m. In panels **C** and **D**, objective 20; scale bar 100 m. *Reprinted with permission from Liu et al.*[14] *Copyright 2010 American Chemical Society.*

from their complex microenvironment that can also be used to detect and differentiate Hodgkin's lymphoma from benign lymph node inflammation.[10]

QD conjugates with streptavidin

Another strategy widely applied is the use of commercially available QD conjugated to streptavidin (QD-SA) which enables a straightforward employment if the biotinylated reagents are available. It normally includes targeting primary or secondary biotinylated antibodies. A 3-step immunofluorescence has been implemented in different human primary tissues.

Fountaine and colleagues have tested the value of QD-SA in identifying a variety of differentially expressed antigens (surface, cytoplasmic and nuclear) in human tissues. They performed a comparison between QD-SA and QD-Ab2 in which QD-SA exhibited three times higher brightness with only one round of amplification (biotin–streptavidin interaction) using identical staining conditions and confocal microscope settings and configurations. Moreover, QD-SA also showed twice as less background than with QD-Ab2. Up to 5 different QD-SA conjugates were used to successfully and simultaneously stain common lymphoid biomarkers to investigate the morphological characteristics of the microanatomy of human lymphoid tissue. The multiplexed staining showed low background, high specificity and high emission intensity, overcoming the major drawbacks encountered with conventional IHC and organic dyes simultaneously labeling tissue sections.[43]

QDs have also become a reliable tool for assessing Her2 levels in BC, which is critical for BC treatment and prognosis. Generally, immunohistochemistry and FISH have been the selected methods for assessing Her2 levels.[44] However they are not efficient for BC prognosis and classification since they present some disadvantages like unstable sensitivity and high discrepancy among different laboratories or interobservers due to its semi-quantitative character.[45] Compared to organic dyes, QDs have proved to exhibit a higher stability and fluorescence intensity. Her2 was labeled in BC tissues with QD525 and FITC both conjugated to streptavidin. Even though QDs absorption spectra are centered at 350 nm, when excited at 460-495 nm (FITC maximum absorption wavelengths), QDs showed a higher fluorescence intensity, photostability and a staining index about 5 times greater than FITC. As expected, even a greater superiority, at least 4-5 fold higher, of QDs labeling was found if excited at 330-383 nm (their optimal excitation wavelength).[46]

By using a 3-step immunofluorescence, comprising primary antibody, biotin labeled secondary antibody and QD-SA, the Her2 status was assessed in 700 patients with invasive BC, validating a new parameter that may reveal BC heterogeneity and novel subtypes of BC with a different 5-year prognosis.[47,48] Among Her2, ER and PR are nuclear receptors also involved in BC development, progression and metastasis. Indeed, positive ER and PR are markers for better prognosis and better response to endocrine therapy. To demonstrate the feasibility and accuracy of QD-based multiplexing technique for simultaneously labeling PR and ER, a comparative study between conventional IHC, QD-based single and double imaging in 17 cases of ductal carcinoma was performed. A good correlation between QD and IHC, and QD-based single and double imaging was found proving that the multiplexing capability of QDs may become a

powerful tool for efficient tumor biomarker assessment in clinical tissues.[49] In another study, the ER, PR and Her2 status was assessed in a larger cohort of patients with invasive BC. Using quantitative QDs-based imaging for the 3 key markers, patients were divided in 5 molecular subtypes which would be helpful for providing more personalized therapies and assessing better prognosis prediction.[50] The use of QDs for the assessment of Her2 levels might have a potential application in clinical diagnosis, since compared to conventional IHC, Her2 detection using QDs was more sensitive, accurate and economic, particularly for IHC (2+) cases. Moreover, QDs have allowed the multiplexed detection of Her2, PR and ER providing a novel subclassification of patients, gaining new insights in cancer prognostic and personalized therapy.

Similarly, in a study in human BC tissue using conventional IHC and QDs, the latter revealed new insights in the heterogeneity of cancer specimens. Chen and co-workers investigated tissue from 75 clinical cases, and QD-IHC showed significantly higher positive rates than conventional IHC, thus pointing towards the QD technique as more suitable to select patients for endocrine treatment. Moreover, the opportunity for a simultaneous staining made it possible to detect simultaneously the expression of different proteins, showing target heterogeneity at different sites, while such distinctions were not noticeable in conventional IHC, and therefore providing new insights on cancer particularities.[51]

Another quantitative analysis using QD-SA was performed to quantify the expression of EGFR in tissue microarrays of 240 BC patients, with the median EGFR total fluorescence area of all the BC patients being 33.08.[52] The prognostic value of EGFR in BC and its relationship with Her2 have been extensively studied for over 20 years; however, there is not a clear agreement on the correlation of these two targets. Therefore, the study of EGFR with novel molecular methods in different patients' populations is needed to provide new insights in this topic.[53] EGFR quantification allowed authors to classify patients into two subgroups, patients with a small EGFR area (<30.51, $n = 113$) and those with a large EGFR area (>30.51 $n = 127$), showing a significant difference in the five-year recurrence-free survival between two groups. QD-based technology has exhibited a good correlation of staining rates ($r = 0.914$) and high agreement of measurement ($\kappa = 0.848$) of EGFR expression compared to conventional IHC with better image quality and sensitivity, allowing to determine the negative prognostic value of EGFR area in patients with Her2+ and lymph node positive BC.[52]

Ki67 is also a key biomarker in BC, whose expression levels have been negatively correlated with BC prognosis.[54] As Her2, it has usually been detected using IHC; however, due to the low sensitivity of the technology, the prognostic value of Ki67 in BC has remained controversial, limiting its clinical applications.[55] Similarly, a more accurate and sensitive method to evaluate the Ki67 expression in BC will help shed light in the molecular significance of this target. In this sense, QD-SA was used for assessing Ki67 expression more accurately and objectively. In fact, Sun and co-workers performed a QD-SA IHC in 108 tissue specimens from BC patients, and it was found that it had a better interobserver agreement for the Ki67 score (2.08% ± 1.54% vs 3.67% ± 2.1%) and a higher sensitivity (80.3% vs 76.1%) than IHC (Figure 4).[56]

In a further example, Yuan and colleagues developed a QD-based quantitative multiple imaging on Ki67 and cytokeratin (CK) in 240 BC patients to better evaluate their influence on BC prognosis. Compared to conventional IHC, the QD-based multiplexed analysis allowed to define a new parameter, Ki67/CK ratio that could be better than Ki67 alone in predicting BC prognosis. According to the results, Ki67/CK relation achieved statistical significance in both lymph node positive and lymph node negative patient's subgroups.[31]

In another comparison of IHC and QDs, Honglei and co-workers labeled caveolin-1 and proliferating cell nuclear antigen (PCNA) in lung cancer tissue from 70 samples of lung

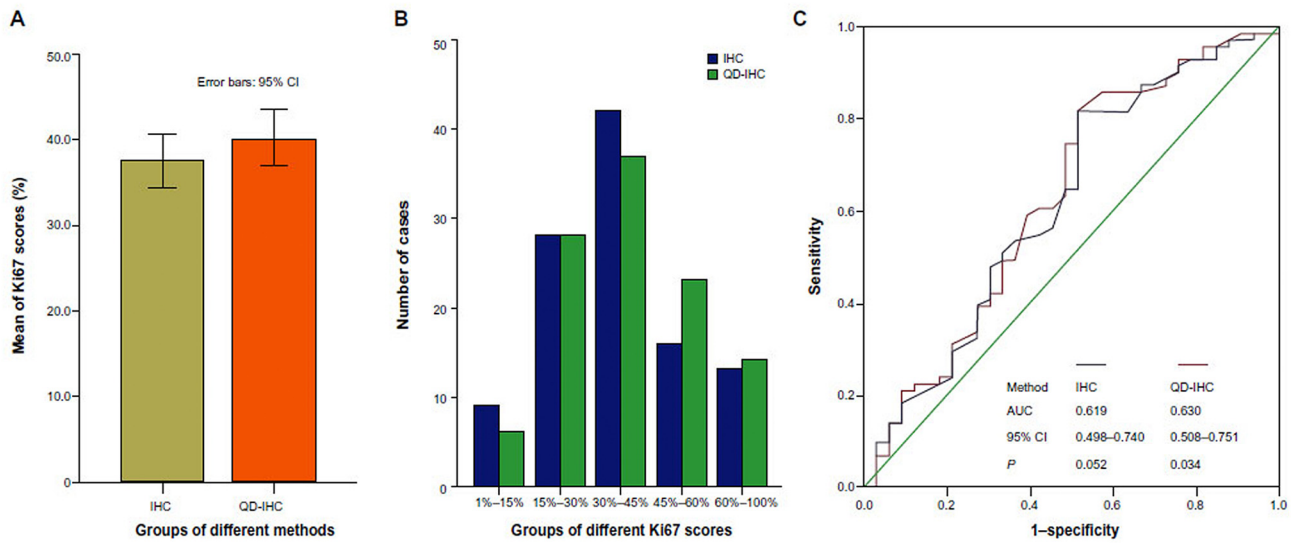

Figure 4. Comparison between QD-IHC and IHC for Ki67 assessment. (**A**) Mean of Ki67 score and (**B**) its distribution in 108 BC patients. (**C**) Ki67 correlation with 5-DFS survival. *Reprinted with permission from Sun et al.*[56] *Copyright 2014 Dove Medical Press.*

carcinomas biopsies. The QDs-IHC showed excellent photostability that allowed for signal observation during the course of 2 months and improved signal-to-background ratio.[57] QDs' improved performance in primary tissue was also assessed by Au and co-workers, who studied Tn antigen in BC tissue microarrays, confirming that QDs showed greater specificity (90% vs 80%) and sensitivity (95% vs 90%) than traditional staining methodologies.[58]

Hu and co-workers also demonstrated that multiplexed QD labeling (mQDL) reveals molecular insights in cancer characteristics at tissue and cellular level with a high degree of sensitivity. In their detailed work, they analyzed prostate cancer specimens with streptavidin-conjugated QDs. They noted that this particular strategy for target labeling provides a higher staining specificity and intensity than a direct conjugation of secondary Ab to QDs (Figure 5). They suggest to perform combinatorial tests to determine the optimal order of addition of antibodies and QDs, as well as using negative controls, internal protein loading controls and when possible, confirming the multiplexed staining with conventional single QD labeling, IHC and WB.[59] In a subsequent study, the mQDL enabled a single cell level analysis detecting 4 biomarkers, reducing sample variability and taking the most out of limited clinical material.[60] Performing similar experiments, multiplexed QD labeling enabled to perform an interracial comparative study studying 6 different targets in the specimens. This cell-based multispectral imaging technique allowed to examine pathways associated biomarkers in the same sample, which demonstrated their usefulness for survival prediction in different population groups.[61] The study showed that the biomarker study of samples derived from PC specimens has the ability to predict patient survival and progression to castration resistance of the disease. In another study about this same cancer, it was also showed how the expression of determined miRNAs correlated with bone metastases and patient survival.[62] QDs were used to determine miRNA in tissue derived from prostate cancer patients. In the same way, QDs have also been employed for Akt-1 quantification in 840 tissue microarray from PC patients improving reproducibility when compared to conventional IHC. Combining QDs immunofluorescence with image deconvolution and computerized image analysis, it was demonstrated that high levels of Akt-1 are an indicator of biochemical recurrence and death, becoming a novel prognostic biomarker in human prostate cancer.[63] Using the same technique, another study studied several human cancer tissues for improved results.[64]

He et al combined both strategies, QD-Ab and QD-SA to investigate the correlation between fibroblastic Cav-1 and LC3B levels in 123 GC specimens. This study revealed that low levels of fibroblastic Cav-1 and high levels of LC3B had a significant predictive value of death. Moreover, they also conjugated QDs with RNA in order to perform a QD-based fluorescent in situ hybridization to establish the influence of EBV infection on the expression of fibroblastic Cav-1 and LC3B. No significant correlation among EBV infection fibroblastic Cav-1 and LC3B was demonstrated. Compared to conventional IHC, QDs multiplexed molecular imaging provided a novel approach to study the heterogeneous expression of proteins in different cells. In this study, a more vivid and easily comprehensible positive correlation between Cav-1 and LC3B was observed using the QDs imaging technology.[65]

While the majority of the research results indicate an imaging superiority of the QDs it is clear that their advantages need to be adjusted and measured in every system. In this sense, Barrow et al performed a comparative analysis of QD staining and conventional IHC staining in tumor sections from Lynch syndrome patients. While QDs exhibited some advantages including multiplexing capabilities, the authors found that IHC performed better in the identification of patients with mutations. Meanwhile, for quantitative IHC the area under the MHL1 ROC curve was 0.872 and the area under the MHS2 ROC curve was 0.832; for quantitative QD-IHC, these were 0.812 and 0.598 respectively.[66]

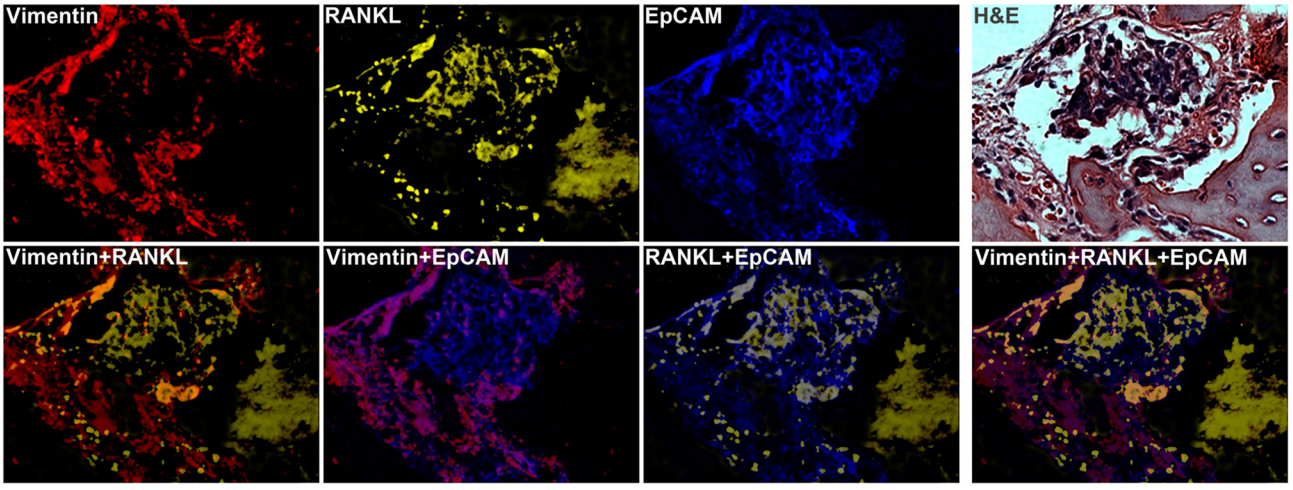

Figure 5. MQDL detects EMT biomarkers in clinical bone tissue specimens. A representative specimen of human prostate cancer bone metastasis co-expressed high levels of epithelial EpCAM, and mesenchymal RANKL and vimentin proteins. ×400. *Reprinted from Hu et al[59] licensed under the Creative Commons Attribution License.*

QD covalent conjugates with primary antibodies

One of the most used techniques with QDs involves attaching antibodies covalently linked to the surface of QDs. While a reliable technique, it often requires a great amount of antibody, making it impractical and expensive. Moreover, Ab linked to the QDs surface may not be properly orientated to recognize the specific target losing bioaffinity. However, the technique has enabled to perform different staining in human primary cells or tissues.

As mentioned before, current methods for BC patient's classification, IHC and FISH, are insufficient to unravel the complex heterogeneity of BC tumor samples.[44] Multi-target-single cell imaging using QD-primary antibody conjugates (QD-Ab) has enabled the quantitative classification of BC subtypes with deeper insights into their heterogeneity. In this sense, Tak and colleagues developed a QD-based quantitative high-content imaging cytometry for BC specimens as a novel approach for BC prognosis and classification. Primary cells from BC biopsied tissues were simultaneously stained with 4 different QD-Ab conjugates (QD525-EGFR1, QD565-Her2, QD605-ER and QD655-PR) to analyze their molecular profiling. Results obtained with QDs were in line with conventional IHC and WB analysis. However, the multiplexed capability of QDs allowed a highly sensitive and more accurate patient classification that is critical for personalized treatment.[12] In another example, the levels of Her2, ER, PR, EGFR and mTOR were simultaneously quantified in BC tissue from patients. QDs emitting at different wavelengths, 525 nm, 565 nm, 605 nm, 655 nm and 705 nm, directly conjugated to primary antibodies were used, suggesting that QDs are well suited for molecular profiling of tumor biomarkers. Results were also compared to conventional single-marker IHC, which multiplexed and molecular profiling capabilities are limited, showing an excellent correlation.[13]

Multiplexed QDs can also be used to properly diagnose and classify renal cancer carcinoma (RCC) patients. QDs were conjugated to antibodies for β-actin and MDM-2 to simultaneously label both biomarkers in cancerous (25) and normal (3) clinical tissue samples. Combining both signals, it was possible to successfully differentiate between clear cells and chromophobe tissue from normal adjacent tissue with 100% of accuracy; meanwhile, separately, no clinical relevance results were achieved. A wider panel of significant biomarkers could improve patients' classification and provide new insights in the molecular pathology of the tumor.[67]

The photostability of QDs directly conjugated to anti survivin Ab was evaluated in head and neck cancer tissue samples. After 5 months, QD-Ab conjugates still showed a high intensity fluorescence; meanwhile, DAPI dye was almost bleached (Figure 6). Moreover, authors also performed a comparison between QDs directly conjugated to primary Ab and commercially available QD-Ab2, showing the latter had higher fluorescent signal. Another staining method that it is not limited by the availability of specific-specie primary Abs or the amount of Ab needed was evaluated. QDs were covalently linked to protein G (QD-PG), an adaptor protein that binds the Fc region of the Ab.[68] However, for multiplexed studies, an additional blocking step to block the free sites on the protein must be performed to avoid cross-linking since the QD-PG–Ab bond is not covalent and Ab could exchange QDs.[69]

Besides cancer, QDs have also been used in the field of infectious diseases. QDs' high stability and low photobleaching compared to organic fluorophores allow long-term imaging for three dimensional reconstructions Human control and *Plasmodium falciparum*-infected erythrocytes were stained with QDs conjugated to anti-band3 (an intrinsic membrane protein of erythrocytes) antibody to study membrane deformation during parasite invasion. Alterations in site of infection of the erythrocyte membrane during parasite infection (marked with Hoechst 33258) were found.[70]

The versatility of QDs allows for their conjugation not only with antibodies but also with other biomolecules of interest such as peptides, oligonucleotides and proteins among others. In this sense, carboxylic acid functionalized QDs were chemically linked to interleukin-13 (IL13). Cytokine functionalized QDs were developed with the aim of identifying exosomes secreted by cancer stem cells and on cerebrospinal fluid (CSF). IL13QD interacted with the interleukin 13 receptor alpha 2 (IL13Rα2) which is correlated with malignancy and it is expressed on exosomes from glioblastoma multiforme (GBM) including glioma stem cells. Results validated IL13QD as an ex vivo marker for glioma stem cells and exosomes that can predict diagnosis and prognosis of

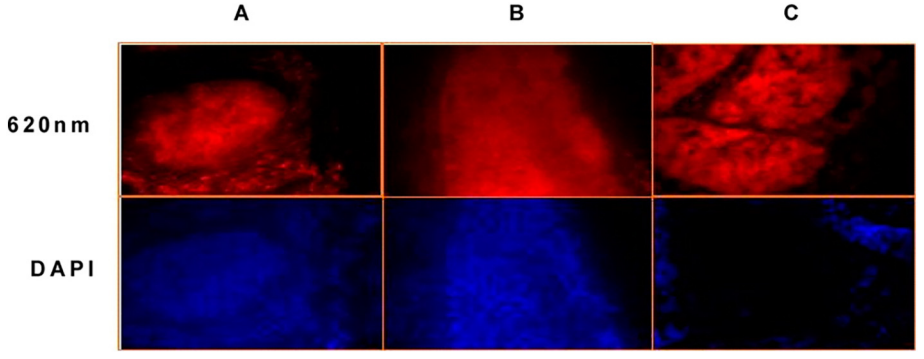

Figure 6. QDs' photostability on human tissues. Images were taken (**A**) immediately, (**B**) 2 weeks and (**C**) 5 months after immunofluorescence was performed. *Reprinted with permission from Xu et al.*[69] *Copyright 2013 American Chemical Society.*

patients and serve as a simple diagnostic tool for early tumor recurrence detection.[71]

Quantum dot safety in primary human models

Similar to other studies with nanoparticles, the potential toxic effects of QDs have been extensively reviewed in numerous studies (Table 2).[18,72–74] However, those performed in primary human cultures and tissues directly derived from patients are not abundant and will be highlighted here. Compared to cell lines, cultures derived from patients are extremely sensitive, providing more consistent results in terms of toxicity[75,76] For this reason, results obtained from toxicity assays in cells derived from patients are more relevant and reflective of the potential toxicity of QDs than those obtained with conventional cell lines.

As pointed out before, the potential toxic properties of QDs cannot be generalized,[3] as QDs differ greatly depending on their size, charge and external functionalization, making it necessary to study QDs with diverse properties.

Amber Nagy and co-workers performed a comprehensive analysis of the biological impact of QDs in primary human lung cells studying the effect of the size, surface charge and particle functionalization.[77] QDs without a shell were synthesized at a size of 3, 5 and 10 nM in diameter and functionalized with either long and short-chain ligands, mercaptoundecanoic acid and mercaptopropanoic acid to have a negative charge and with amino undecanethiol or cysteamine to have a positive charge. The rationale behind using primary pulmonary epithelial cells was to study the potential risk of particle inhalation. Positively charged QDs showed greater toxicological impact than negatively charged QDs, producing greater necrosis, in which QDs with longer ligands exerted a greater response. The production of reactive oxygen species showed to be more size dependent, and particularly more pronounced for negatively charged QDs. Regarding apoptosis and proliferation, QDs did not show to directly affect these parameters independently of size, charge or ligand functionalization. In a subsequent study that exhaustively studied the genotoxicity on QDs in these primary cells, it was however found that both positively and negatively charged QDs (functionalized with cysteamine and mercaptopropionic acid respectively) did induce DNA rupture. Therefore, the authors suggest that cytotoxic studies must be complemented with genotoxicity evaluation in order to better comprehend the impact of QDs in primary cells. Finally, a gene expression analysis was performed to investigate molecular events related to cellular effects where it was found that positively charged QDs could be producing mitochondrial damage by affecting mitochondrial membrane potential.[77,78]

In order to assess QDs biocompatibility and determine if they can be used in vivo, Wang and co-workers investigated the effect of QDs cytotoxicity in human amniotic membrane-derived mesenchymal stem cells (hAM-dMSCs) at different concentrations (0.75, 1.5 or 3 μg/mL) and incubation times (1, 2 or 4 days). For this purpose, different biological approaches, analysis of morphological and growth features, MTT cytotoxicity assay and expression of specific surface antigens (CD29 CD44 CD90 and CD105) were used. hAM-dMSCs were efficiently and safely labeled at 0.75 μg/mL, with a cell viability greater than 80% and with a similar expression of the specific surface antigens profile.[79]

To assess the cytotoxicity of polymerized histidine-formaldehyde coated QD nanoparticles that could be accumulated in the kidney, Li and co-workers studied their cytotoxicity in human primary renal proximal tubule cells. They found that primary cell lines were more sensitive to toxic materials than regular cell lines and showed inter-donors variation that must be controlled to have reliable and reproducible results.[76]

The effects of UVB exposure in QDs cytotoxicity in primary keranocytes were studied, in order to mimic interactions that the nanoparticles could experience while penetrating the epidermis. Dihydrolipoic acid coated QDs were incubated with keranocytes

Table 2
Summary of results obtained in the safety evaluation of QDs in primary human cells and tissues.

Reference	QD type	Primary cells or tissue type	Results found
[77–78]	3, 5 and 10 nm QDs without shell, functionalized with mercaptoundecanoic acid, mercaptopropionic acid, amino undecanethiol and cysteamine.	Lung cells	Greater toxicity found on positively charged QDs, and QDs functionalized with longer ligands Mitochondrial damage exerted by positively charged QDs No effect in apoptosis and proliferation Both positively and negatively charged types of QDs produce genotoxicity
[79]	Not specified	Amniotic membrane-derived mesenchymal cells	QDs show good biocompatibility at different concentrations and incubation times
[79]	Polymerized histidine-formaldehyde coated QD	Primary renal proximal tubule cells	Primary cell lines are more sensitive to toxic materials than regular cell lines and present interdonor variations
[80]	Dihydrolipoic acid coated QDs	Keranocytes	No significant results in UVB radiation induced cytotoxicity
[81]	Tri-n-octylphosphine oxide, poly(maleic anhydride-alt-1-tetradecene) co-polymer (TOPO-PMAT)-coated QDs	Liver cells	Particle uptake different from cell lines Inflammatory response observed through pro-inflammatory cytokines/chemokines

with or without UVB radiation; however, no significant results in the cytotoxicity examined were found.[80]

Tri-n-octylphosphine oxide, poly(maleic anhydride-alt-1-tetradecene) co-polymer (TOPO-PMAT)-coated QDs' cytotoxicity was examined in primary human liver cells together with HEPG2 cells. Firstly, they found differences of particle uptake between the primary cells and the cell lines (Figure 7), confirming the importance of utilizing clinical samples in order to better assay the effects of QDs cytotoxicity in humans. While no acute toxicity signs were found, an inflammatory response was observed with some cytokine level elevation.[81]

Cellular uptake and tracking

Finally, in order to be used as potential tools for in vitro and in vivo cell tracking studies (Table 3), QDs' efficient labeling, high stability and cytotoxicity must be investigated. Understanding the mechanism of QDs uptake is a key matter for QDs to be used as diagnostic, imaging a therapeutic nano-agents. Different methods have been developed for QDs delivery into cells, such as electroporation,[82] microinjection,[83] reversible cytoplasmic membrane permeabilization,[84] and QD conjugation with protein transduction domains or cell penetrating peptides.[85,86] Interestingly, experimental evidence suggests that QDs can enter cells via different endocytic routes depending not only on QD physicochemical properties, such as size, charge or surface modification, but also on the cell type.[87] Carboxyl-coated QDs have been proved to enter cells via clathrin-mediated endocytosis[88–90] but also same QDs can penetrate cells via lipid raft/caveolin-mediated endocytosis.[87,91] Other studies have proved that functionalized QDs with different biomolecules of interest such as peptides,[92] small molecules[93] or growth factors[87] enter cells via clathrin-mediated endocytosis. After internalization, some QDs accumulated in endosomes while others were transferred to lysosomes for degradation or transported to the membrane and exocytosed.[93,94] In fact, QDs have also been used to study the process of exocytosis, being more suitable than conventional synaptic vesicles dyes since their larger size prevents QDs to escape from vesicles during the formation of the exocytosis fusion pore.[90,95,96]

Dr. Genicio and co-workers have assessed the viability of primary cultured human limbal epithelial cells (HLECs) extracted from cadaveric corneas that were transplanted onto decellularized human corneo-scleral rims. QDs exerted no apparent toxicities and they could be monitored for 2 weeks after transplantation. Authors claim that QDs could be used to track HLECs, cells that have shown potential for the therapy of limbal stem cell deficiency.[97] In the same line of research, Duncan and colleagues have developed a novel method using QDs to validate the quality and barrier functionalization of HLECs obtained from human corneas to be used in transplantation. Epithelial cells were incubated with two different sizes of QDs (QD525 and QD655) in either normal calcium containing

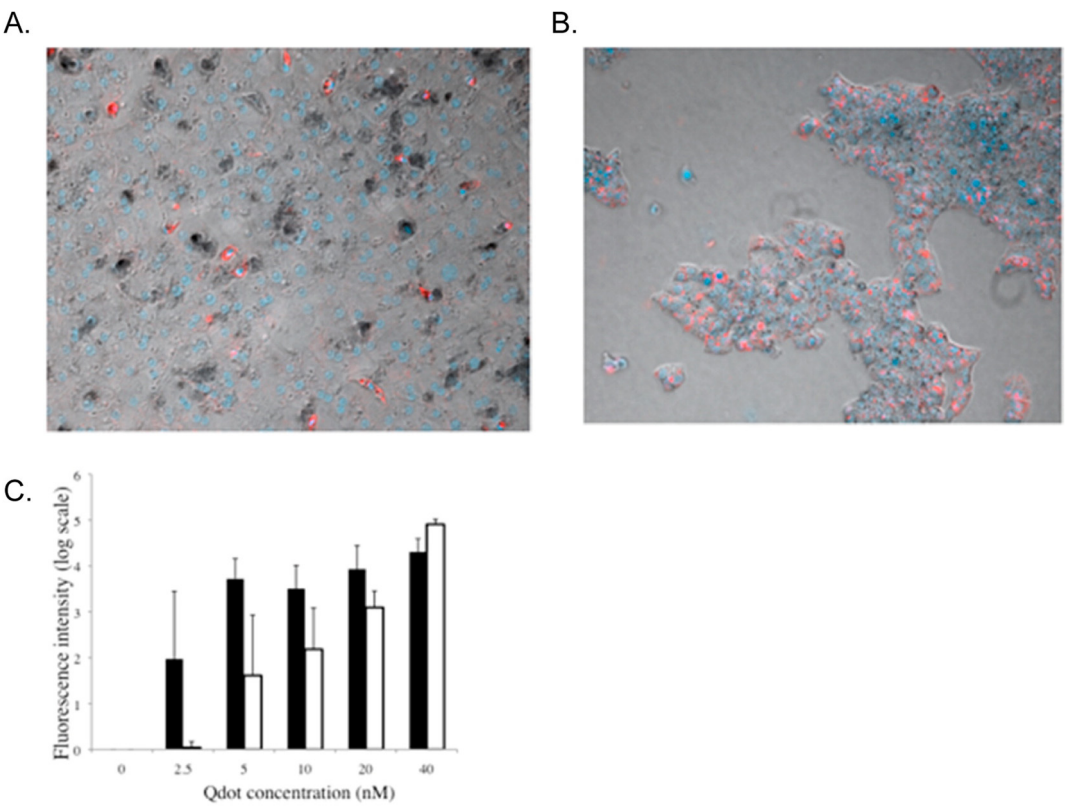

Figure 7. QD uptake comparison between primary human liver cells and human conventional cell lines. (**A**) QD uptake by primary human liver (PHL) cells, (**B**) QD uptake by human liver cell line HepG2 and (**C**) QD uptake quantification of PHL cells (white) and HepG2 (black). *Reprinted with permission from Smith et al.*[81] *Copyright 2012 American Chemical Society.*

Table 3
Summary of cellular uptake and tracking in primary human cells and tissues with QDs.

Reference	QD type	Primary cells or tissue type	Results found
[97]	QD-Tracker peptide conjugate	Primary limbal epithelial cells from cadaveric corneas	Cells monitorization with existing clinical equipment for 2 weeks after transplantation
[98]	Carboxyl coated QDs	Primary limbal epithelial cells from corneas	Evaluation of cultured epithelial cell sheet barrier Qualitative and quantitative morphology data were obtained, together with the localization of specific areas of damage
[100]	QD-Tracker peptide conjugate	Primary cells from umbilical cord blood Mononuclear cells from bone marrow of chronic myeloid leukemia patients	Cells were labeled for 2 weeks and dividing cells were tracked for up to 4 divisions 95% of control cells had visible QD fluorescence, showing the potential to track hematologic cells through cell division and differentiation
[101]	QD-Tracker peptide conjugate	Primary blood mononuclear cells	Parameterization of fluorescence signal dynamics
[102]	Gelatin nanospheres incorporating carboxyl quantum dots	Induced pluripotent stem cells 3D cartilage tissue	Multiplex staining with imaging reliability without cytotoxicity or affected cell proliferation Homogeneous labeling for 4 weeks
[104]	QD-Tracker peptide conjugate	Post-mortem eye tissue	Labeling of different cell regions of the trabecular meshwork
[107]	QD peptide conjugate	Human MSCs from bone marrow	Size dependent internalization, dispersion, instability and leakage found
[109]	Carboxylic acid QD	hMSCs	Cytoplasm penetration of QDs, non-toxic in concentration up to 16 nM
[113]	Carboxylic acid QDs	hMSCs	Efficient labeling of hMSC without apparent toxicity
[114]	QD-Tracker peptide conjugate	amniotic stem cells	Cell sex has an influence on the uptake of QDs
[115]	Carboxyl QDs and QD-Tracker peptide conjugate	hMSCs	In vivo three dimensional tracking observed for 8 weeks
[110]	QD conjugated to anti-mortalin Ab	hMSCs	Confirmation of intercellular communication and QD transfer from hMSC to BC cell line
[111]	Carboxyl coated QDs	hMSCs from skin	Study of optimal uptake conditions, release, toxicity and functional effects
[112]	Carboxyl coated QDs	hMSCs and primary metastatic BC cells	Demonstration of QD transfer from nano-engineered MSCs to BC cells
[116]	RGD peptide QD conjugate	hMSCs	Effective labeling during proliferation and differentiation
[117]	RGD peptide QD conjugate with β-cyclodextrin	hMSCs	Custom QDs promote osteogenic differentiation and enable hMSCs long term tracking
[118]	Polyethyleneimine coated QDs to form Gene-complexed bundled NPs	hMSCs	DNA binding and transfection capabilities
[119]	Amino-PEG-QDs bundled in nanogels	hMSCs	QD allowed to visualize the internalization of sunflower-type nanogels into hMSCs
[120]	Carboxylated QDS	Cadaveric subjects	Identification of the distribution and long-distance pathways in the human body

medium or medium with a calcium chelator (EGTA) that resulted in the disruption of tight junctions. A larger amount of both QDs was observed in the damaged cell sheets since a deeper and more ubiquitous QDs penetration is permitted. Cell viability was assessed and no significant negative effect of QDs or EGTA was found. Besides providing a novel technique for evaluating HLECs function, this method also offers qualitative and quantitative data of HLCEs morphology and allows to localize specific areas of damage.[98]

As cytotoxicity, the potential use of QDs for long-term labeling or tracking studies is subjected to its stability in living cells. QD fluorescence can be reduced due to surface coating biodegradation, oxidation of the nanoparticle core or dose dilution through cell division.[99] Again, performing these experiments in human primary cells or tissues will give valuable information compared to the one extracted from results in cell lines.

CD34+ cells isolated from human umbilical cord blood were incubated with QDs tracking labeling kits (Q-Tracker) for 10 h to demonstrate the potential of QDs to track cells through cell division and differentiation. For cell division studies, CD34+ cells were also labeled with PKH26 a validated dye for tracking the number of division, and sorted by FACS to obtain a uniform fluorescent population. After 4 days, 30% of undivided cells were QD positive; meanwhile, QDs could be seen in 10% and 2% of cells that had undergone through one or two divisions respectively. QDs retention during CD34+ cells differentiation was also demonstrated since after 5 days, QDs were positive in about 1% monocytes. Finally, mononuclear cells isolated from human bone marrow from acute and chronic myeloid leukemia (AML or CML) or controls were incubated with Q-Tracker. Around 90% and 55% of AML and CML patient's cells were labeled with QDs respectively. Moreover, 95% of control cells from different lineages including, myeloid lineage, erythroid lineage, lymphocytes and megakaryocytes, had visible QDs by fluorescence microscopy. In this sense, authors demonstrated that QDs could be used to track hematologic cells through cell division and differentiation, which are critical aspects in hematology to help answer the origin of cells of interest.[100]

Summers and colleagues performed a systematic study of QDs fluorescence stability in primary blood mononuclear cells (MNCs) isolated from peripheral blood from healthy adults. The work allowed to parameterize fluorescent signal dynamics using analytical mathematical expressions. A rapid initial decay in QDs fluorescence was observed during the first 6 h followed by QDs signal stabilization.[101]

Murata and co-workers developed a labeling method of human induced pluripotent stem (iPS) cells-derived 3D cartilage tissue using QDs. In this sense, 3 types of QDs emitting at different wavelengths, 525 nm, 605 nm and 705 nm, and iron oxide nanoparticles (IONP) were internalized in gelatin nanospheres with octa-arginine cell penetrating peptide, for multimodal cell imaging. These imaging probe carriers didn't show cytotoxicity or affected cell proliferation.[102] Two different protocols were tried, a direct labeling that only targeted the cells residing in the surrounding site of cartilage tissue; and a dissociation and labeling method that homogenously labeled the 3D cartilage pellet and the cell sheet for at least 4 weeks. The multiplexing capability of QDs allowed an enhancement of the visualization and imaging reliability.[103]

Different fluorescent tracers including QDs bioconjugates and QD labeled microspheres were perfused into anterior segments from human donor eye post-mortem tissue to label and characterize flow regions of the trabecular meshwork (TM). Since elevated intraocular pressure is the primary risk for glaucoma, the characterization of the flow regions of the TM may have significant impacts on the design and delivery of treatments for the disease. By varying the size of the fluorophores (QDs modified with a HIV-TAT, amine functionalized QDs or amine and carboxylate fluorospheres) different cell regions of the TM were labeled which might be especially beneficial for targeting drug therapies.[104]

Human mesenchymal stem cells (hMSCs) show promising applications in the field of regenerative medicine as cell-based therapy of various diseases, mainly cancer.[105,106] MSCs could become potential drug delivery systems to transport therapeutic molecules directly to cancerous tissues. Several studies summarized below have studied MSCs safety, efficacy and mechanism of action using QDs to efficiently translate stem cell therapy to the clinic.

Ranjbarvaziri and co-workers studied these properties labeling hMSCs from bone marrow with commercially-available QDs conjugated with a cell penetrating peptide, with different emission peaks at 525 nm, 585 nm and 800 nm. hMSCs effectively internalized QD525, QD585 and QD800 observing a size dependent incubation time. However, QD525 showed a higher dispersion inside the cytoplasm of cells which resulted in a loss of stability.[107] According to Selevertov and colleagues, QD525 selective clearance from hMSCs may be due to autophagy activation or to loss of the QD hydrophilic coating.[108] Carboxylic acid-functionalized QDs emitting at 625 nm were nontoxic to hMSCs at 16 nM and efficiently and quickly taken up by the cells. QDs were mainly located in the cytoplasm and did not penetrate the nucleus minimizing the potential genotoxicity of QDs that could bind DNA.[109]

To serve as drug delivery systems hMSCs need to transfer their cargo to the cancer cells. Using QDs conjugated to antimortalin Ab, the intercellular communication between hMSCs and BC was confirmed. hMSCs and BC cells were previously incubated with two different QD conjugates, QD655 and QD585 respectively. After QD uptake, cells were co-cultured in monolayer. Within 48 h of co-culture, the amount of QD double positive BC cells increased exponentially. Moreover, authors also established that direct cell-to-cell interactions mediated intercellular QD transfer from hMSCs to BC cells. This study supports the potential role of hMSCs as drug delivery for cancer therapies.[110]

Saulite and co-workers have studied the cellular uptake, release toxicity and functional effects of carboxyl-coated QDs in hMSCs from skin to prove the potential of MSCs as vehicles for tumor-targeted QD delivery.16 nM QD concentration and 6 h incubation time were selected as the optimal QD conditions for the uptake experiments since cells were 99% QD-positive and hMSC viability was not affected. An exhaustive analysis of the uptake pathway of QDs in the presence and absence of serum (to avoid protein non-covalent adhesion on the surface of QDs

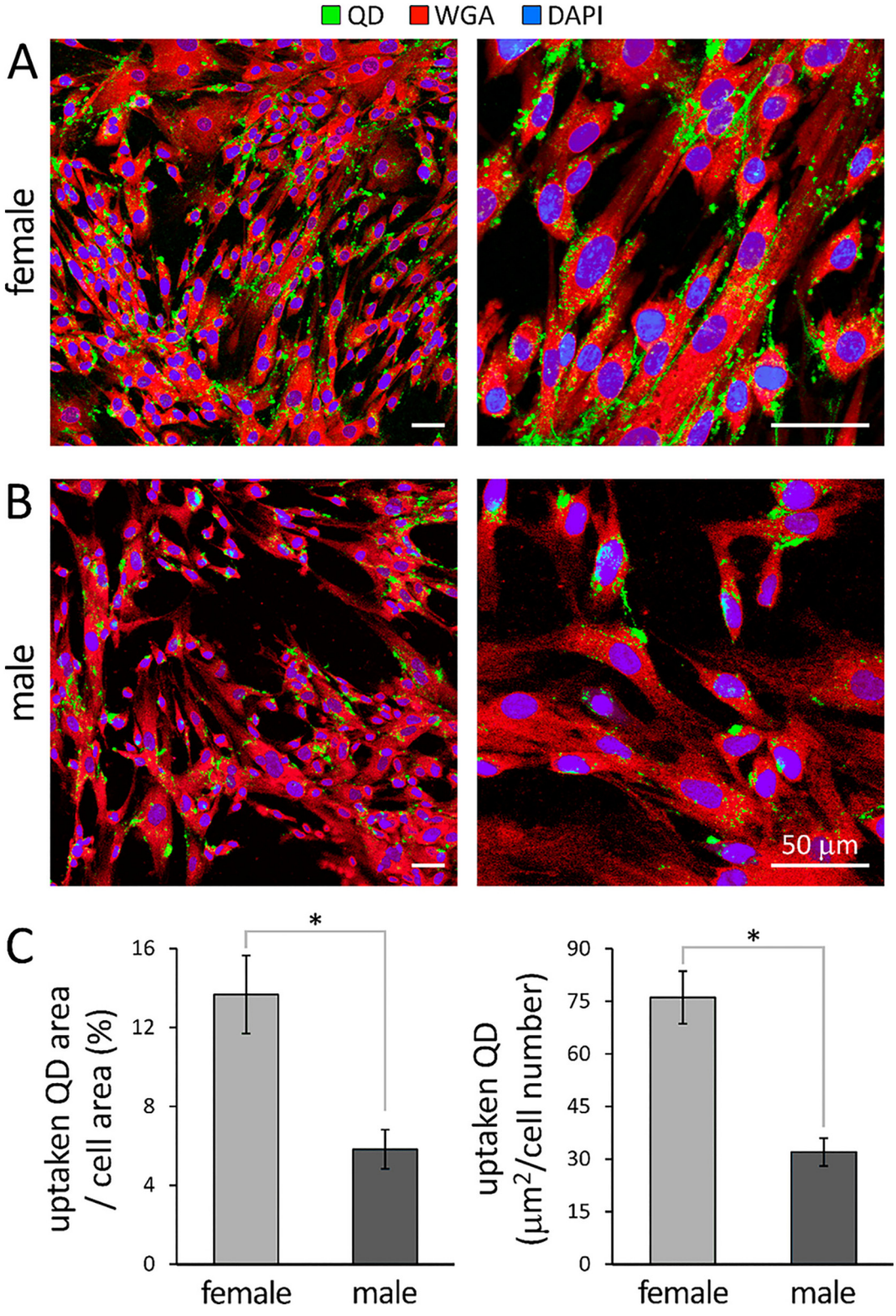

Figure 8. QD uptake differences in female and male hMSC. QD-IHC imaging of (**A**) female hAMSCs, (**B**) male hAMSCs and (**C**) quantification of QD fluorescence, proving a significant increase in QD uptake in female versus male cells. Scale bar represents 50 µm in all panels. *Reprinted with permission from Serpooshan et al.*[114] *Copyright 2018 American Chemical Society.*

which might trigger the entrance of QDs via ligand-receptor mechanism) was examined. In complete medium, QDs uptake in hMSCs was mediated through clathrin-mediated endocytosis; meanwhile, in serum-free conditions, the clathrin and caveolin/lipid raft-mediated endocytosis pathways were involved. Interestingly, under serum free conditions, hMSC showed a higher cellular uptake. After 6 h QDs were mainly localized in early endosomes and later transferred to lysosomes. Consequently, after 24 h of incubation, a 30% and 40% decrease of fluorescence signal was observed in cells incubated in complete medium and serum-free conditions respectively, which could reflect QD excretion or degradation. A larger decrease was observed after 48 h in serum-free cultivated cells. Finally, QDs did not exhibit any negative effects on the proliferation of MSCs and neither did alter hMSCs' capacity to differentiate into adipocytes, chondrocytes and osteocytes.[111] In a subsequent study, the QD transfer from nano-engineered hMSCs to BC cells via P-glycoprotein excretion was demonstrated in a 3D cell co-culture model. Two different cell lines were co-cultured among with hMSCs, a primary and a metastatic BC cell line, the latter exhibiting a 4-fold enhanced uptake. Such difference in QDs' uptake efficiency might be due to different endocytosis pathways. While QDs enter MCF7 cells through both phagocytosis and clathrin/caveole-dependent endocytosis, MDA-MB-231 cells internalized QDs through caveole-dependent pathway.[112]

In another work, Kundrotas and co-workers analyzed the uptake and distribution of carboxyl QDs in hMSC isolated from bone marrow. Commercially available carboxyl QDs with an emission peak at 625 nm were used. In general, QDs were able to label hMSC with no apparent toxicity, with slightly different QDs localization depending on culture density.[113] Sex-dependent differences in QDs uptake were found in male and female human amniotic stem cells (hAMSCs), the latter showing a QD uptake about 2 times higher (Figure 8), and in primary fibroblast isolated from the salivary gland of healthy male and female donors. In contrast to hAMSCs, male primary fibroblast exhibited a 6 times greater QD uptake than female cells. To better understand the effect of cell sex on QDs uptake, the localization of the clathrin heavy chain was determined by super-resolution microscopy STORM images, before and after QD incubation. Significant differences in accumulation on the cell surface and colocalization of clathrin heavy chains with QDs were found between male and female hAMSCs, suggesting that QD uptake occurred through clathrin-mediated endocytosis in male cells, but not in female cells. Similarly, an increase concentration of clathrin heavy chain was found in male fibroblast than female fibroblast. These differences might be due to disparity in secreted factors and the cytoskeleton and therefore should be further studied to enable effective clinical translation of QDs.[114]

In the same type of cells, Rosen and co-workers confirmed that long-term cell tracking was highly optimized with the use of QDs. The increased photostability and improved signal to background ratio enabled tracking for 8 weeks after delivery and enabled a 3D reconstruction of their distribution.[115]

QDs conjugated to RGD peptide have also effectively labeled hMSC, targeting selected integrins on the cell membrane. Additionally, these QD-peptide bioconjugates labeled hMSCs during proliferation and differentiation into multilineage differentiation.[116] Li and co-workers also confirmed that a multifunctional QD nanoparticle containing RGD conjugated peptide together with siRNA and a small molecule constituted a valuable tool in the study of regenerative medicine with two objectives: hMSC long-term tracking and osteogenic differentiation induction.[117] Another key aspect regarding stem cells applications in regenerative medicine drug delivery was examined by Yang et al. Polyethyleneimine QDs bundled nanoparticles were prepared to measure gene delivery efficiency into hMSC, showing DNA binding and transfection capabilities.[118] Another subsequent strategy studied by the same team analyzed sunflower-type nanogels that, differently from QD-encapsulated nanogels, were suitable as a delivery vehicle.[119]

QDs have also been used as fluorescent tracers to visualize long-distance extravascular pathways in cadaver subjects. Carboxylated QDs of 10 nm diameter and QDs microspheres

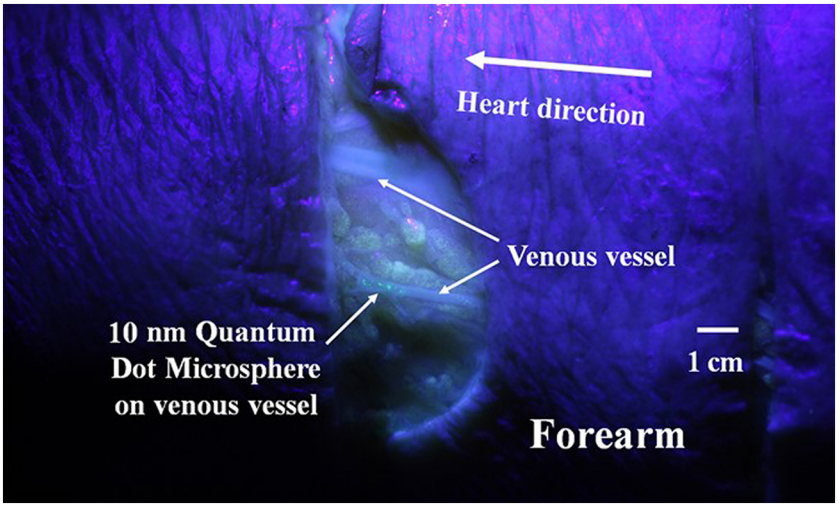

Figure 9. QDs on a venous vessel. After 2.5 h of compression on the heart, the 10 nm QD microspheres from the right thumb can be found on a venous vessel in the forearm. *Reprinted from Li et al*[120] *licensed under the Creative Commons Attribution License.*

of 100 nm were injected in the right thumb of the subjects and after 2.5 h of continuous chest compressions, both types of QDs were found on the distal phalanx of the right thumb. Moreover, the smaller QDs could also be found in some venous vessels in the forearm of the subjects (Figure 9). These data combined with additional MRI experiments performed in healthy volunteers allowed to identify the distributions and structures of long-distance pathways in the human body.[120]

Conclusions

The proliferation of commercially available QD probes positively provides for an increasingly generalized use of these luminescent materials for several purposes. However, their use in primary human samples derived from patients including cells, tissues and post mortem specimens is still scarce. Given the benefits of using these biological samples that provide better models of disease and personalized diagnostics more experiments in this area are needed. The unique photoluminescent properties of QDs offer great advantages that can be of great use to understand molecular routes and pathological events in specimens derived from patients. Moreover, the enhanced photostability and multiplexing capabilities enable taking the most out of these limited primary samples.

Overall, the most abundant studies belong to the field of oncology, due to specimen availability and the valuable information that tissue analysis offers. However, with the development of techniques involving iPSC derived from patients a new reality offers the possibility to study cells from patients, sometimes with diseases in which tissue extraction is not an option until post-mortem, such as neurological disorders. We anticipate that merging both cutting edge techniques will allow for a deeper understanding of disease in a personalized manner.

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