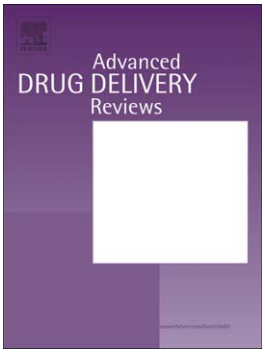

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Multifunctional quantum dots-based cancer diagnostics and stem cell therapeutics for regenerative medicine

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Abstract

A field of recent diagnostics and therapeutics has been advanced with quantum dots (QDs). QDs have developed into new formats of biomolecular sensing to push the limits of detection in biology and medicine. QDs can be also utilized as bio-probes or labels for biological imaging of living cells and tissues. More recently, QDs has been demonstrated to construct a multifunctional nanoplatform, where the QDs serve not only as an imaging agent, but also a nanoscaffold for diagnostic and therapeutic modalities. This review highlights the promising applications of multi-functionalized QDs as advanced nanosensors for diagnosing cancer and as innovative fluorescence probes for *in vitro* or *in vivo* stem cell imaging in regenerative medicine.

Key words:

Quantum dots; FRET; Microfluidic device; Cancer diagnosis; Stem cell labeling; Cationic liposomes; Cell-penetrating peptides; Stem cell therapy; Cell transplantation

Contents:

1. Introduction

The development of semiconductor quantum dots (QDs) has been heading toward an interdisciplinary field with bioassays and bioimaging technologies [1-3]. It offers significant advantages over those that the previous methods, which have relied on conventional organic fluorophores or fluorescent proteins. The unique and useful optical properties of QDs have been explored such as broad absorption spectra, narrow emission spectra, high quantum yields, long fluorescent lifetime, size-tunable photoluminescence, and exceptional photostability with a strong resistance to photobleaching [4-7]. The optoelectronic characteristics of QDs arise through the systematic transformation in the density distribution of the electronic energy levels as a function of the size of the QDs [8]. As a result, the nature of the QDs surface plays a key role in their optical behavior. The ability to modify the surface of QDs with biomolecules or other polymers through various conjugation methods underpins their versatility.

QDs have been incorporated into various biological assays for the reversible detection and quantification of biomolecules. Such assays often focus on the use of distance-dependent fluorescence resonance energy transfer (FRET) [9.10] and multiplexed bioanalysis with multiple colors [11,12]. These techniques can be introduced for applications such as immunoassays [13,14], molecular diagnosis [15,16], clinical assays [17-19], and cellular analysis [20,21]. Many kinds of QDs-based biological assays have been demonstrated in bulk solution or on solid substrates [22]. A more competitive platform is microfluidic device. The device is able to handle the extremely small quantities of fluid with multiple analytical samples transported through the microfabricated channels [23]. It can provide opportunities for further removal or replacement of QDs-based sensing components [24].

When applying QDs to biological imaging and cellular studies, the toxic nature of cadmium-containing QDs remained a major concern. However, with the recent advances in the development of surface modifications of QDs and cadmium-free QDs, the potential toxicity of cadmium is no longer a problem for *in vitro* and *in vivo* imaging studies. The cellular uptake of QDs can be modulated by their size [25], shape [26-28], and surface functionalization [29]. In fact, the use of multicolor QDs for stem cell imaging is probably the most important and clinically relevant application for regenerative medicine in the immediate future. Despite the enormous potential of QDs

in therapeutics, the fundamental information on the interaction between QDs and therapeutic cells [30] is relatively limited. This review thus aims to outline the beneficial properties presented by QDs, along with important advances in their biological applications for cancer diagnostics and stem cell therapeutics. Particularly, the observation of the QDs-mediated cellular responses, such as cellular uptake and intracellular behavior of QDs, will provide insights into the nanoparticle design and the therapeutic efficacy for regenerative medicine.

2. Quantum dots-based sensing and detection

2.1. Quantum dots

QDs stand out as one of the most exciting research tools in chemistry, physics, and biology (Fig. 1). These inorganic fluorescent nanocrystals typically comprise periodic groups of CdSe and CdTe or InP and InAs semiconductor materials. For the semiconductor nanocrystals, the energy levels are quantized due to the quantum-confinement effect [31,32], and their spacing can be controlled by the crystal sizes. This effect leads to the superior optical properties of QDs, such as narrow, symmetric, and size-tunable emission spectra. Also, the broad excitation spectra of QDs enables multicolor fluorescent applications. Compared with organic fluorophores or fluorescent proteins, QDs show 10 to 100 times brighter fluorescence and 100 to 1,000 times higher fluorescence stability against photobleaching. These benefits of QDs facilitate long-term monitoring of intermolecular and intramolecular interactions in living cells and tissues. Consequently, much interest has been focused on the exploration of QDs for biomedical applications.

Among the array of synthetic routes devised for the preparation of biocompatible QDs, the coating of the CdSe core with the ZnS layer is indispensable [3,20]. Passivation by the ZnS layer protects the CdSe core from oxidation, and reduces the toxicity of the CdSe core from leaching out to the surrounding solutions. It also enhances the photoluminescence yield. Although the synthesis of QDs has been performed directly in aqueous solution, QDs themselves have little specificity for the aqueous nature of the biological environment [33,34]. For example, the ZnS-coated QDs are only soluble in nonpolar organic solvents. Altering the surface properties of QDs mainly rely on the conjugation with biological molecules such as aptamers [35], antibodies [36], oligonucleotides [37], and peptides [38] to gain the biological affinity [39].

As a result of the conjugations of QDs, they have achieved optimal stability, monodispersity, crystallinity, solubility, and biocompatibility in the fields of diagnostic and therapeutic research. For example, within a certain range of concentrations, CdSe/ZnS shows good biocompatibility with human amniotic mesenchymal stem cells [40]. The effect of QDs size and poly(ethylenimine) coating on the labelling efficiency with stem cells has been also reported [41]. Furthermore, a peptide-coated QDs can mimic cellular transport mechanism in stem cells through endosomal escape [42].

2.2. Fluorescence resonance energy transfer

In terms of the fluorescence application to monitor binding interactions or conformational changes of biomolecules, FRET phenomenon, the non-radiative energy transfer between an excited state fluorophore (donor) and another fluorophore (acceptor) through long-range dipole-dipole coupling, has been recognized as a quite useful detection scheme in various bioanalysis [43]. The utility of this detection scheme is explained that it generates fluorescence signals sensitive to the changes in 1 to 10 nm range. More specifically, the FRET-based transduction using QDs as donors has become a popular approach for assay development. Their broad absorption spectrum in the UV region can be used advantageously to avoid the direct excitation of acceptor dyes. As a result of the introduction of multiple pathways for energy transfer, the FRET efficiency can be enhanced by the QDs. The transduction strategy can be also arranged using QDs and dyes. For example, many QDs have the opportunity to interact with a single acceptor dye, or multiple dyes can interact with a single QD.

QDs-based FRET probes have also been reported, including QDs-conjugated hybridization probes for the preliminary screening of siRNA sequences [44] and an ultrasensitive DNA nanosensor with a single QD [37,45], which benefit from the use of the unique photophysical properties of QDs and their conjugates. Recent work in our laboratory [46] has demonstrated the feasibility of using QDs as a nuclease-tolerant FRET probe. The streptavidin-coated QD binds to the biotinylated DNA end (Fig. 2A), and the FRET-inducing electrostatic coupling and the structural changes to the QD-DNA conjugates can be detected (Fig. 2B) based on the conjugation of the QDs with YOYO-3 dye-intercalated DNA. The QD-DNA conjugate and the tolerance properties induced by dye binding are thought to be notable due to the utilization of QD-conjugated functional materials in living cells or tissues. Transduction via FRET

also makes it possible to employ multiple methods for data analyses. For example, ratiometric detection is often used for differences in assay preparation among multiple analyses and instrumental drift. The narrow symmetrical emission profile of QDs facilitates the deconvolution of acceptor signals for the recovery of absolute signals [47].

2.3. Microfluidic devices

Microfluidic system is based on the microfabricated structures to control or manipulate fluids constrained in a small space [23,24]. The potential to be used in biological applications is a major driving force behind the rapid development of this system [48-51]. They are primed to be powerful tools to provide the basis for the detection of biomolecules with small amounts of reagents and simple operation [48]. In addition, the large surface area-to-volume ratio and mass transport by non-diffusive means offers the transduction of analytes within seconds to minutes.

Microfluidic devices have been known to facilitate single-molecule measurements in combination with QDs [52]. Such approaches are useful to determine patterns and distributions that may otherwise be masked by ensemble averages [53]. Single-molecule studies are usually performed by monitoring a fixed volume of solution. Analytes are allowed to diffuse into this fixed volume in order to be detected. A continuous-flow system, as found in capillaries and microfluidics, ensures that multiple analytes are moved across the detection volume, increasing the probability of detection. For example, a mismatch repair protein complex that slides while maintaining continuous contact with DNA can be visualized by QDs in real time [54]. The DNA-binding proteins are engineered with epitope tags and labeled with antibody-coupled QDs. The motion of the proteins is observed on the single DNA molecule that is anchored and elongated in a microfluidic device. Using similar protein labeling methods with QDs, several kinds of specific sequences related to genetic or epigenetic changes of genomic DNA molecules have been detected for optical mapping applications [55]. Moreover, the mapping method is successfully applied to the detection of DNA methylation at a single molecule level [56].

On the other hand, recent advances in a microfluidic system developed by our group (Fig. 3A and B) allow for the visual observation of nucleolytic cleavage for the direct screening and detection of a specific sequence in genomic DNA [57]. By using DNA

molecular tagging with QD, it is possible to track the motion and position of a single DNA molecule (Fig. 3C and D) gaining access to a restriction enzyme on the microchannel surface. This kind of experimental approach will help enable applications for ultra-sensitive, high-speed and small-volume bioanalytical measurements.

3. Cancer diagnosis by quantum dots

3.1. Protein and nucleic acid

Early diagnosis of cancers is at the forefront of cancer research. Cancer biomarker-based *in vitro* assays are useful for the screening and diagnosis of cancer, as well as for monitoring the response to therapy. Many proteins are considered as useful diagnostic markers of various cancers [58-60]. They are also the targets for basic biomedical research. Since the protein biomarkers are often present at very low concentrations, analytical methods with low detection limits are required for the early diagnosis of cancers [61]. QDs have proven to be applicable for the sensitive detection of cancer biomarkers such as ovarian cancer [62], breast cancer [63], and prostate cancer [64]. More recently, two tumor biomarkers, α-fetoprotein and carcinoembryonic antigen in human serum, have simultaneously been detected by a QD-based nanosensor [65].

Nucleic acids are another type of tumor marker for various cancers. QDs are also very useful in the detection of nucleic acids, especially in a multiplexed format. For example, a multicolor optical coating for biological assays comprises embedding different-sized QDs into polymeric microbeads at precisely controlled ratios. These were designed as a DNA hybridization system using oligonucleotide probes and triple-color encoded beads. The coding signals can identify different DNA sequences [66]. Moreover, a solid-phase FRET assay using immobilized QDs as donors can detect two target sequences simultaneously [22]. This assay showed that over 50% of the analyte's signal readout was obtained even in bovine serum and with a large excess of non-complementary genomic DNA as background noise.

More recently, a single-quantum dot-based nanosensor for specific miRNA detection has been developed [67]. Among the numerous analytical approaches for miRNA detection, most suffer from problems such as non-specificity and low sensitivity. To overcome these problems, two-stage exponential amplification reactions and single-QD-based nanosensors were fabricated. The detection limit of 0.1 aM miRNA

was achieved, and even single-nucleotide differences between miRNA family members could be distinguished.

3.2. Cancer cells

Sensing the interaction between drug-carrying vehicles and cell membrane is the primary requisite for a successful diagnostic process, where the diseased cells are first located, following by subsequent cellular uptake and release of a therapeutic agent into the cytosol or nucleus of cells [69]. Especially for cancer research and therapy, QDs have been utilized as imaging probes [3,20] for the recognition of specific cell types and tissues in the clinical settings [68]. For example, a static immunostaining of cellular targets with QDs has been demonstrated and shown to be both brighter and more photostable than comparable organic fluorophores [17]. In particular, QD-peptide conjugates can specifically target the tumor vasculature in mice [38]. A PEG coating has reduced the reticuloendothelial clearance in the *in vitro* histological results. An ABC triblock copolymer-coated QD probe has also been developed to target and image prostate cancer [36]. For *in vivo* studies, the tumor site can be actively probed by the antibody-conjugated QDs and imaged in living animals. The large size and immunogenicity of antibodies sometimes affect their pharmacological behavior. Alternatives are small ligands such as peptides [39] or aptamers [35] that are employed to conjugate QDs.

A QDs nanoprobe has been developed to sense glioma cells based on the overexpression of the extracellular matrix glycoprotein, tenascin-C [70]. Tenascin-C is involved in tissue remodeling, and plays a role in the invasion of glioblastoma into the surrounding brain tissue. The QDs were targeted to tenascin-C by a single-stranded DNA aptamer. Non-cadmium-containing QDs have also been shown to be able to label cells fluorescently [71]. For example, phospholipid micelle-encapsulated silicon QDs could be conjugated to transferrin and taken up by pancreatic cancer cells. The concentration of silicon QDs applied to these cells was not toxic, because there was 95% cell viability after 24 h.

Since the cellular uptake of exogenous material generally occurs through internalization mechanisms [72], QDs are passively uptaken via non-specific endocytosis along the migratory pathway of human mammary epithelial tumor cells. Moreover, unmodified QDs have been used as an alternative marker over gold

nanoparticles for phagokinetic tracking to monitor cell motility as a potential assay for cancer metastasis [73]. The use of a transfection agent such as liposomes or micelles can assist the delivery of QDs. Polymer- or ligand-modification of the QDs is usually more specific and efficient than non-specific endocytosis alone [74,75]. A general observation is that the endocytosed QDs are often trapped in endosomes and lysosomes. They can be visualized as punctate fluorescence staining. This plays an important role in tracking the spatiotemporal behavior of the cells.

4. Stem cell labeling by quantum dots

4.1 Stem cell labeling

Regenerative medicine is expected to overcome the shortage of donated organs, donor site morbidity and immune reactions. Many kinds of stem cells, such as induced pluripotent stem (iPS) cells [76,77], embryonic stem (ES) cells [78,79], and some kinds of somatic stem cells, including bone marrow-derived stem cells [80,81] and adipose tissue-derived stem cells [82,83], have been discovered and may be useful in numerous applications for regenerative medicine [84-86]. To ensure the safety and maximum therapeutic effects of regenerative medicine, analyses of the behavior, accumulation and condition of transplanted stem cells *in vivo* have become increasingly important [87,88]. However, the conventional *in vivo* imaging modalities used in clinical practice are not sufficient for the analysis of transplanted stem cells [89,90]. Fluorescent imaging (FI) with the prominent fluorescence properties of QDs is expected to detect the transplanted stem cells *in vivo* with higher sensitivity in comparison to other imaging modalities [91,92]. Thus, "stem cell labeling technology using QDs" and *"in vivo* fluorescent imaging technology for visualizing transplanted stem cells labeled with QDs" are essential for analyzing the transplanted stem cells.

There are generally thought to be two methods that can be used to label stem cells with QDs; the conjugation of QDs with a stem cell surface [93] and the transduction of QDs into a stem cell [91,94]. The conjugation of QDs with the cell surface is associated with some problems, such as a reduced accumulation rate of stem cells in tissues/organs and the separation of QDs from the stem cell surface during circulation in the body. The transduction of QDs into the stem cell may overcome these problems; however, the cytotoxicity of the QDs is very high and the transduction efficiency is very low when using physical stimulus methods, such as ultrasonic transduction [95] and

electroporation [96,97]. In contrast, the labeling of stem cells with QDs using chemical modification methods, such as cationic liposomes, cell-penetrating peptides, and high molecular nano-carrier (polymer micelles) was reported to be useful [98-100], because these molecules have been used in clinical applications for the transduction of DNA and proteins into cells.

4.2 Cationic liposomes

QDs are unable to label stem cells with high efficiency because of their low rate of interaction with the cell membrane, so chemical modifications using cationic liposomes are expected to be useful for the stem cell labeling application of QDs [101,102]. Some cationic liposomes such Lipofectamine® (Life technologies), COATSOME® (NOF corporation), and ScreenFect™A (Wako) are commercially available, and are mainly used for gene transfection. These cationic liposomes can interact with negatively-charged QDs (especially COOH-conjugated QDs), then the cationic liposomes rapidly enclose the negatively-charged QDs through electrostatic forces [94,103].

In fact, when cationic liposomes and negatively-charged QDs were mixed at the optimal mixture ratio in culture medium and then were added to stem cells in the culture medium. The QDs could be transduced into the stem cells through endocytosis within a few hours, and were maintained in the cytoplasm near the nucleus by escaping the exocytotic mechanism [104] (Fig. 4A). The transduction efficiency was very high, and no severe cytotoxicity was identified [105,106] (Figs. 4B and C). In addition, QDs labeling with cationic liposomes did not affect the stem cell characteristics, such as their potential for self-renewal and their multilineage potential [94] (Fig. 4D). This labeling method is simple and relatively rapid, and so appears to be useful for stem cell labeling with QDs.

However, high concentrations of cationic liposomes induce the death of stem cells. The cell membrane is generally negatively-charged, so the cell membrane structure is unstable and destroyed by the interaction with strong positively-charged cationic liposomes. The validation of the optimal concentration of cationic liposomes is important and necessary to make it possible to label various kinds of stem cells without adverse effects [102].

4.3 Cell-penetrating peptides

Cell-penetrating peptides (CPPs), also known as protein transduction domains (PTDs), are expected to be useful for the stem cell labeling application of QDs, because several transduction domains can deliver a large size-independent variety of molecules into cells, including proteins, peptides, antisense oligonucleotides and large metal beads [107]. Representative CPPs include the Tat protein of human immunodeficiency virus (HIV-1) [108,109], VP22 protein of herpes simplex virus [110] and Antennapedia (Antp) homeoprotein of *Drosophila* [111], and all of these CPPs possess arginine- and lysine-rich sequences. In addition, poly-arginine (PolyR), especially 8~11-arginine peptides, and poly-lysine have been shown to exhibit an even greater efficiency in the delivery of several peptides and proteins [112,113] (Fig. 5A).

Most studies have reported that these CPPs were conjugated with negatively-charged QDs (CPP-QDs) through chemical or electrostatic forces, and the condition of the QDs surface was dramatically changed to make it positively-charged. In a case of chemical binding, the CPPs and QDs were mainly chemically-bonded by the amide bonding of the amino group of CPPs with the carboxyl group of the negatively-charged QDs [106,112]. Chemically-bound CPPs-QDs are very stable; however, the synthesis and purification of CPPs-QDs involves a great deal of time and effort. On the other hand, in the case of electrostatic binding, the CPPs and QDs were conjugated by the positively-charged amino acids of CPPs binding the negatively-charged functional groups of the QDs [114,115]. These electrostatic-bound CPPs-QDs were stable in cell culture medium, and the production of CPPs-QDs is simple and quick in comparison to chemical binding methods.

Indeed, in that case, CPPs-QDs could be transduced into stem cells through micropinocytosis within several hours, and were maintained in the cytoplasm near the nucleus by escaping exocytosis (Fig. 5B). Macropinocytosis occurs independent of clathrin-mediated and caveolin-mediated endocytosis, and the size of particles that can be uptake is more than 1 μm, and the process requires dynamin GTPase activity [116]. Similar to the complexation with cationic liposomes, the transduction efficiency for micropinocytosis was very high, and there was no severe cytotoxicity observed (Fig. 5C). The CPPs-QDs did not affect the stem cell characteristics such as the self-renewal or multilineage potential [117,118] (Figs. 5D and E). In addition, the CPPs do not generally induce the death of stem cells at high concentrations, as occurs when cationic

liposomes are used for transfection [91]. Thus, this labeling is also expected to be utilized for the stem cell labeling of QDs. Moreover, a previous study showed that iPS cells could be labeled with QDs using CPPs at high efficiency, and iPS cells labeled with QDs maintained their undifferentiated state and pluripotency [119] (Figs. 6A and B). Therefore, CPPs are expected to be useful molecules for stem cell labeling with QDs. However, validation of the optimal concentration is important and will necessary to determine the best way to label various kinds of stem cells without adverse effects [120].

5. *In vivo* fluorescence imaging of transplanted stem cells labeled with quantum dots

5.1 *In vivo* fluorescence imaging

In vivo fluorescence imaging systems, which can detect and analyze the fluorescence or emission from the body, have been developed for small animals such as mice and rats [88]. The Maestro™ (PerkinElmer) Clairvivo OPT (SHIMADZU) and IVIS Imaging System (PerkinElmer) are representative instruments (Fig. 7A). These systems generally have an integrated ultrasensitive cooled CCD (charge-coupled device) camera, and can detect the weak fluorescence and emission *in vivo*. The number of photons from the fluorescence or emission can be counted, and the fluorescence or emission intensity can then be quantitatively determined. In addition, these fluorescence or emission data can be combined the data from other modalities, such as X-ray CT (computed tomography) [121], MRI (magnetic resonance imaging) [122,123], SPECT (single photon emission computed tomography) and PET (positron emission computed tomography) [124,125].

These systems can be used for *in vivo* fluorescence imaging of transplanted stem cells labeled with QDs. However, there are some major problems that prevent the successful *in vivo* fluorescence imaging of transplanted stem cells at high resolution, which include the strong scattering, absorption and autofluorescence derived from the whole body. In order to overcome these problems, fluorescence probes that can absorb the excitation light and emit strong fluorescence in the near-infrared region (about 700~900 nm) in the "Biological Optical Window" to decrease the scattering, absorption and autofluorescence derived from the body, are strongly desired [126-128] (Fig. 7B). Some QDs showing strong fluorescence in the near-infrared region have been developed and are commercially available, so QDs are expected to be useful for *in vivo* fluorescence

imaging of transplanted stem cells (Fig. 7C).

Moreover, the autofluorescence derived from normal food must be considered [129]. In fact, autofluorescence of mice given normal feed was detected from the gastrointestinal tract by excitation in the red or near-infrared region. To diminish the impact of this effect on the results of the *in vivo* fluorescence imaging, the mice were given feed not including fluorescent components (alfalfa-free feed) for at least one week. The autofluorescence intensity of the alfalfa-free feed was reported to be about 10-fold lower than that of normal feed (Fig. 7D).

5.2 Subcutaneous transplantation

In vivo fluorescence imaging of subcutaneously transplanted stem cells labeled with QDs into mice has been frequently reported [88,130]. In fact, the fluorescence intensity derived from QDs could be detected and analyzed quantitatively by using an *in vivo* fluorescence imaging system after the subcutaneous transplantation of different numbers of stem cells labeled with QDs into the back or other sites of mice. Subcutaneously transplanted stem cells labeled with QDs could be detected clearly at the level of several thousand cells over a period of about one week, and the fluorescence was QD dose-dependent [91] (Figs. 8A and B). However, the stem cells labeled with QDs could proliferate rapidly, so the sensitivity of detection was decrease in inverse proportion to the proliferation of the stem cells.

Moreover, the multiplex imaging of subcutaneously transplanted stem cells has been reported by utilizing the fluorescence characteristics of QDs [91,131]. In fact, stem cells were labeled with different kinds of QDs emitting fluorescent light from 525 to 800 nm, then the stem cells could be detected in different colors using excitation of the same wavelength at high resolution [131] (Fig. 8C). The availability of QDs emitting fluorescence in the near-infrared region is relatively low for subcutaneous transplantation, because visible light can enter through the skin to some extent. This technology is expected to be useful for *in vivo* fluorescence imaging of different cell populations included in regenerative tissues and organs at the same time.

5.3 Intravenous and other transplantation

Stem cell therapy via intravenous transplantation has been expected to be useful in clinical applications for some diseases of the lungs, liver and pancreas [132-134].

Adipose tissue-derived stem cells [ASCs], bone marrow-derived stem cells, hematopoietic stem cells and progenitor cells derived from these stem cells have all been used for stem cell therapy [84-86,135]. *In vivo* fluorescence imaging of intravenously-transplanted stem cells labeled with QDs may make it possible to trace the transplanted stem cells *in vivo* and analyze their rate of accumulation into specific tissues or organs [88,89].

In a previous study, adipose tissue-derived stem cells labeled with QDs were intravenously transplanted into mouse models of emphysema or acute liver disease, and then the location and the rate of accumulation of stem cells in major organs, such as the heart, kidneys, lungs, liver and spleen, were investigated [129,136]. In the emphysema model, the transplanted ASCs were observed in the lungs at one and four hours after transplantation, and more ASCs remained in the lungs with emphysema compared with the lungs from normal mice [136] (Fig. 9A). In the model of acute liver disease, the transplanted ASCs were found to accumulate more in the liver when there was simultaneous administration of heparin compared to when the ASCs were transplanted alone [129] (Figs. 9B, C, and D).

In these cases, QDs with near-infrared fluorescence were useful for the *in vivo* fluorescence imaging of transplanted stem cells, because near-infrared fluorescence can be seen through the skin and organs at high efficiency. In fact, intravenously transplanted ASCs labeled with QDs800 (fluorescence peak at 800 nm) could be detected in the lungs and liver even without laparotomy; whereas transplanted ASCs labeled with QDs655 (fluorescence peak at 800 nm) could not be detected [129] (Figs.9B, C, and D).

On the other hand, bone marrow-derived stem cells labeled with QDs were transplanted in the ipsilateral striatum of a rat model of cerebral infarct, then the impact of the timing and stem cell dose on the therapeutic effects were determined by *in vivo* fluorescence imaging of transplanted stem cells labeled with QDs [137]. Neural stem cells labeled with QDs were transplanted into the striatum contralateral to the ischemic hemisphere, then the therapeutic effects were confirmed by *in vivo* imaging of transplanted neural stem cells labeled with QDs. The findings of that study suggest that the *in vivo* fluorescence imaging of transplanted stem cells labeled with QDs may assume increasing importance in association with the development of stem cell therapy for regenerative medicine.

6. Recent progress and views in overcoming the problems caused by using QDs

Three major problems caused by using QDs, such as assurance of the safety, realization of high functionality, and barrier of national regulation, appear to be resolved for the clinical application to stem cell therapy and regenerative medicine. Firstly, the assurance of safety is thought to be the most important problem. Much safer elements against several kinds of cells including iPS cells or bodies should be selected as the constituent elements of QDs to overcome this problem. Secondly, the realization of high functionality enable us to expand the extent of the application of QDs such as the detection of state changes of stem cells. Finally, the actual situation of the barrier of national regulation on regenerative medicine by using stem cells including iPS cells should be considered in each country.

CdSe and CdTe semiconductor materials have been generally used as the core of QDs, however the these elements were reported to show the cytotoxicity at low concentration. Thus, the novel QDs based on silicon or $AgInS_2$ cores not including Cd, Se, and Te elements have been already developed, and these QDs were reported to have very low cytotoxicity [138-140]. On the other hand, QDs-based sensing mechanisms such as FRET and multiple colors are thought to be useful for the detection of the state changes of stem cells. However, there are little information and reports on these technologies, thus the early development of these technologies is strongly expected [9-12]. Moreover, clinical research environment have been improved in recent years. New three laws associated with regenerative medicine have been established especially in Japan, so the barrier of national regulation on regenerative medicine have become lower all over the world. Collectively, QDs will actually be able to apply for stem cell therapy and regenerative medicine in the future with the further improvement and development.

7. Conclusions and future directions

Organic fluorophores have been widely used as fluorescent probes in conventional biometrics; however the research on QDs for biometrics has expanded greatly in recent years. The recent findings suggest that QDs can be used to detect and analyze objects which conventional biometrics cannot detect. In this review, we described the fluorescence properties of QDs, and then introduced QDs-based biometrics technologies, the “Bio-sensing” technology, Stem cell labeling technology and *In vivo* fluorescence

imaging technology for transplanted stem cells. These technologies will likely contribute to regenerative medicine, especially stem cell therapy, which requires high-sensitivity imaging at the cellular level.

The potential clinical applications of QDs have been expanded by the development of considerably low cytotoxicity QDs that do not include cadmium (Cd) or selenium (Se) [115], as well as the development of the second near infrared region (1,000 ~ 1,500 nm) fluorescent QDs with strong permeability into the body [141,142]. In fact, clinical trials based on the data obtained from basic research have been started in the U.S.A. [143], and research and development are becoming increasingly important. Moreover, the evolution of QDs including the development of hybrid materials of QDs and other functional molecules probably enables the diagnosis and therapy of stem cells *in vivo* at the same time, an approach termed "*in vivo* Theranostics".

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Figure captions

Fig. 1. Schematic of a major negatively charged QD.

Fig. 2. A nuclease-tolerant FRET probe.

A: Schematic of the FRET between the QD donor and the DNA-intercalating dye (YOYO-3) acceptor. The QD emission overlaps the absorption spectrum of the dye, suggesting that an efficient FRET between the QD donor and the YOYO-3 dye acceptor can take place. B: An electrophoresis analysis of the FRET probe for the effect on DNA digestion. A yellow circle shows the undigested FRET probe by a restriction endonuclease (EcoRI).

Fig. 3. A microfluidic molecular tracking system.

A: Schematic design for the time-series analysis of ongoing processes in the enzymatic reaction through the detection of single-molecular movements. B: Layout of the molecular tracking chip. Enzymatic reactions are done in a conventional microfluidic device, consisting of a polydimethylsiloxane (PDMS) microstructure and a syringe pump. A single QD was connected to one DNA molecule as a tracking dye. Enzymes are immobilized on the bottom surface of a microchannel, and QD-tagged DNA molecules are flown in the channel. The fluorescent images during the event are captured by a total internal reflection fluorescence (TIRF) microscope. C: Video monitoring in action at the enzyme area. The flow keeps the motion of DNA molecule constant, and a restriction enzyme (Apa I) on the surface changes the motion. D: A trajectory of QD-tagged DNA represented as x coordinate, y coordinate and fluorescence intensity. Dashed line shows the control with no enzyme immobilized.

Fig. 4. Quantum dots labeling using poly-cationic liposome (Lipofectamine®) for imaging of adipose tissue-derived stem cells.

A: Morphology of ASCs and fluorescence of the QDs655 (0.8 nM) observed by phase-contrast microscopy. B: Cell viability of ASCs labeled with QDs655 using Lipofectamine®. C: Proliferation rate of ASCs labeled with QDs655 using Lipofectamine®. D: Adipogenic and osteogenic differentiation of ASCs labeled with QDs655 (0.4 nM).

Fig. 5. Quantum dots labeling using cell penetrating peptides (octa-arginine: R8) for imaging of adipose tissue-derived stem cells.
A: Constitutional formula of arginine molecule and the information of normal chain R8. B: Optimal formation of QDs and R8 for labeling ASCs. C: Cell viability of ASCs labeled with QDs655 using R8. D: Proliferation rate of ASCs labeled with QDs655 using R8. E: Adipogenic and osteogenic differentiation of ASCs labeled with QDs655 (0.4 nM) using R8.

Fig. 6. Maintenance of undifferentiated state and multipotency of iPS cells labeled with QDs using R8.
A: Morphologies and fluorescence images of 1 day after EB formation of non-labeled iPS cells and iPS cells labeled with QDs using R8 (phase contrast, red fluorescence derived from QDs655, and green fluorescence derived from GFP). B: Teratoma formation of iPS cells labeled with QDs using R8 after four weeks of the injection of labeled iPS cells to the nude mouse. a: Obtained histological sections were stained with hematoxylin and eosin. b: Nerve-like structures, c: cartilage-like structures, d: gut epithelium-like structures, e: adipose-like structures, f: glomerulus of the kidney-like structures.

Fig. 7. Biological window in near-infrared region and comparison of the fluorescence intensity between normal with alfalfa-free feed.
A: Biological window in near-infrared region (about 700-900 nm) B: Fluorescence spectra of QDs655, QDs705, and QDs800 in the culture medium. C: *In vivo* imaging fluorescence system. a: The Maestro™ (PerkinElmer), b: Clairvivo OPT (SHIMADZU), c: IVIS Imaging System (PerkinElmer) D: Comparison of the fluorescence intensity between normal with alfalfa-free feed. a: *In vivo* fluorescence image of mouse fed on normal feed. b: Fluorescence images of normal and alfalfa-free feed. c: Fluorescence intensity of normal and alfalfa-free feed.

Fig. 8. Detection and multiplex imaging capability of QDs in subcutaneous transplantation.
A: *In vivo* fluorescence images of mouse subcutaneously transplanted stem cells (0.5, 1.0 and 3.0 × 10^5 cells)labeled with QDs655 (0.8 nM) using R8 into the backs of the

mice after 1 h, 1, 2, 5 and 7 days. B: Bar graph of the changes of fluorescence intensity at the number of stem cells labeled with QDs655 using R8 for 7 days. C: *In vivo* fluorescence Image of mouse subcutaneously transplanted stem cells labeled with QDs525, 565, 605, 655, 705 and 800 into the back of the mice after 10 min with a single excitation light source.

Fig. 9. *In vivo* and *ex vivo* imaging of stem cells labeled with QDs using R8 after intravenous injection.

A: *In vivo* fluorescence images of mouse transplanted stem cells labeled with QDs800 using R8 through the tail vain into mouse after 10 min. B: *In vivo* fluorescence images of mice with acute liver failure after transplantation of stem cells labeled with QDs655 using R8 in combination without heparin (a) or with heparin (b) without laparotomy. C: *In vivo* fluorescence images of mice with acute liver failure after transplantation of stem cells labeled with QDs800 using R8 in combination without heparin (a) or with heparin (b) without laparotomy. D: *Ex vivo* fluorescence images of five organs in mice with acute liver failure after transplantation of stem cells labeled with QDs800 using R8 without heparin (a) or with heparin (b), and ratio of the fluorescence intensity (RFI) of five organs (c).

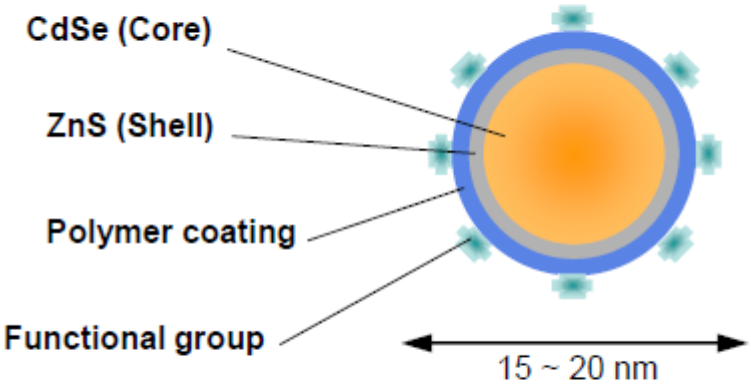


Figure 1

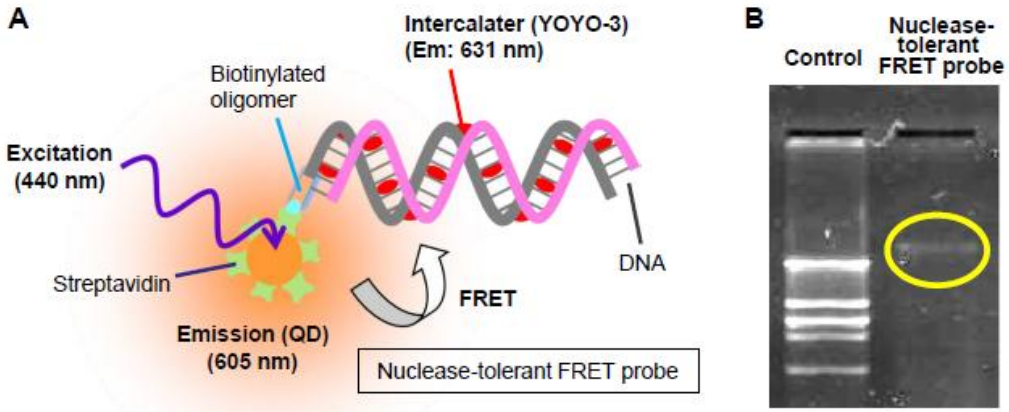


Figure 2

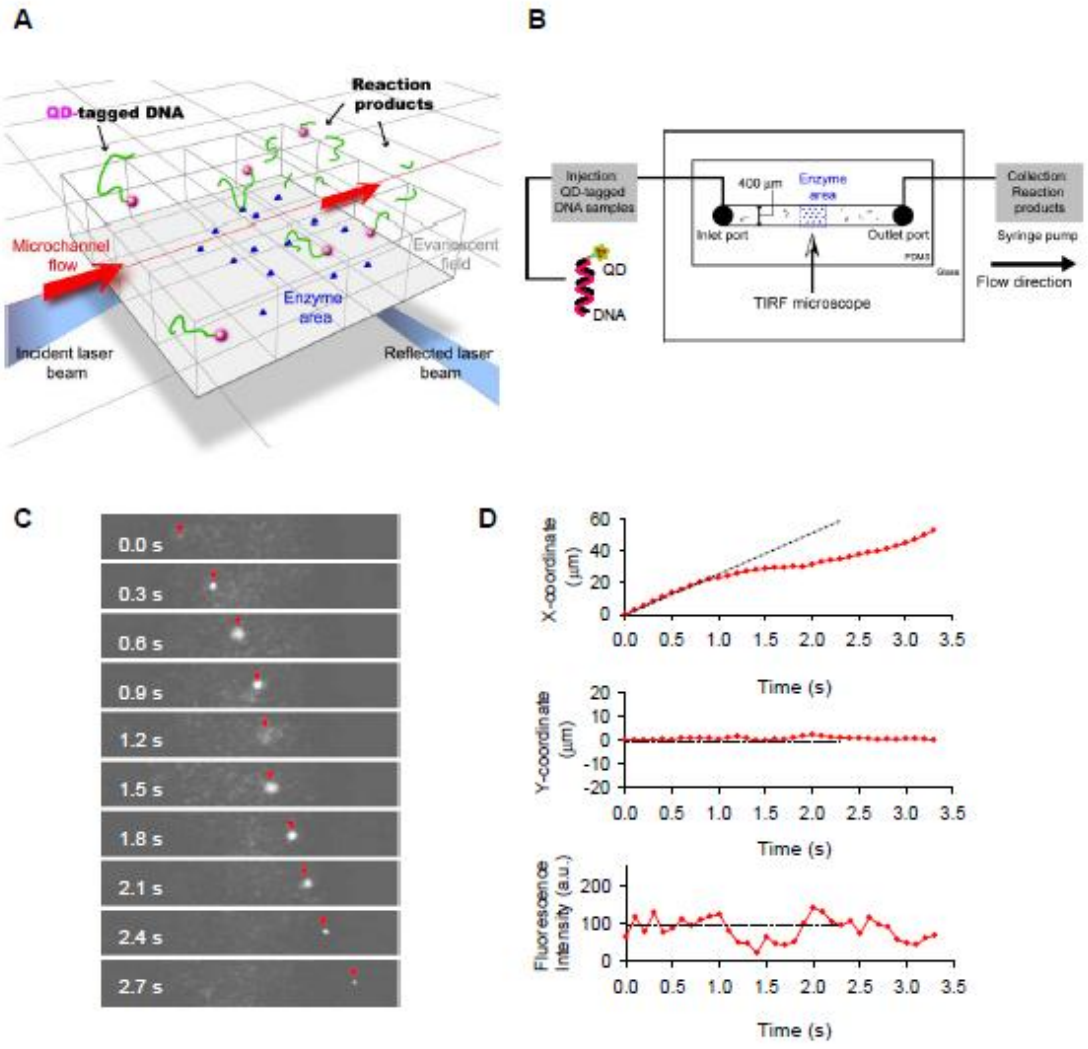
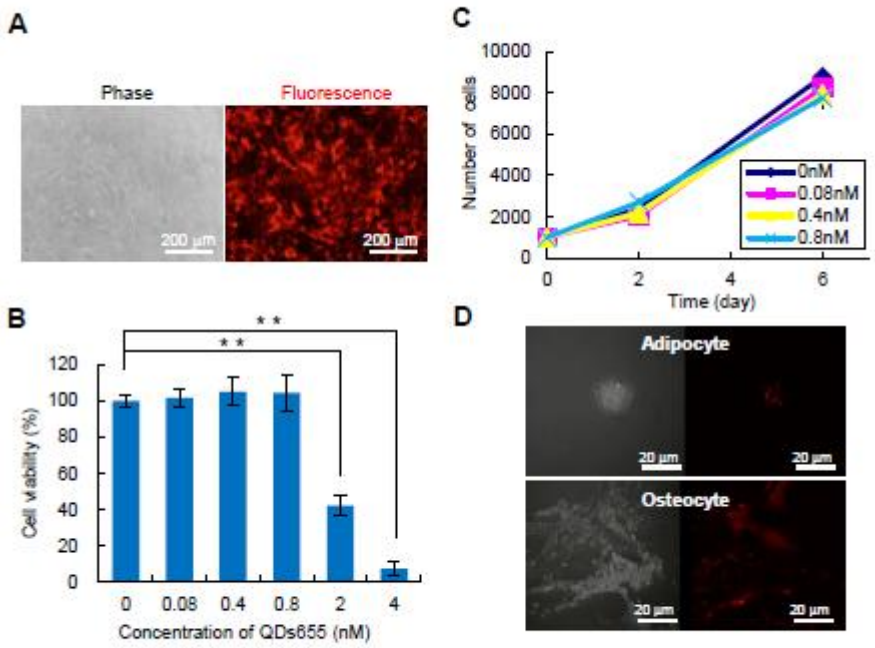


Figure 3



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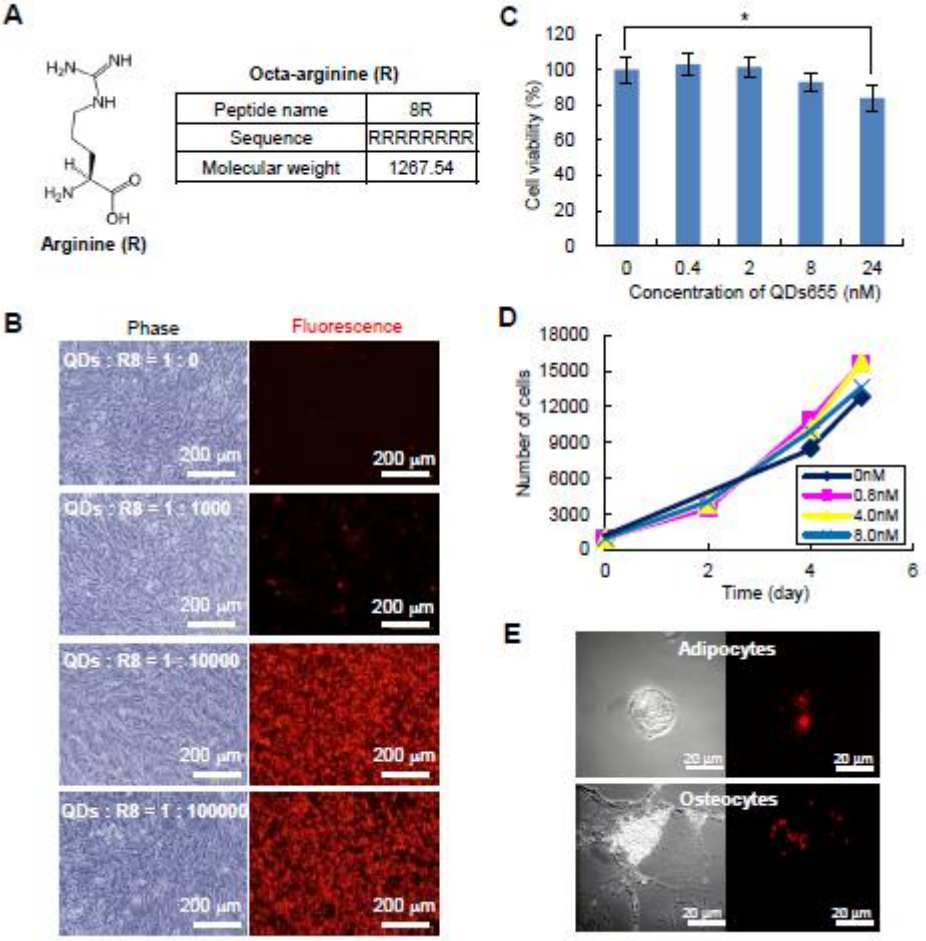
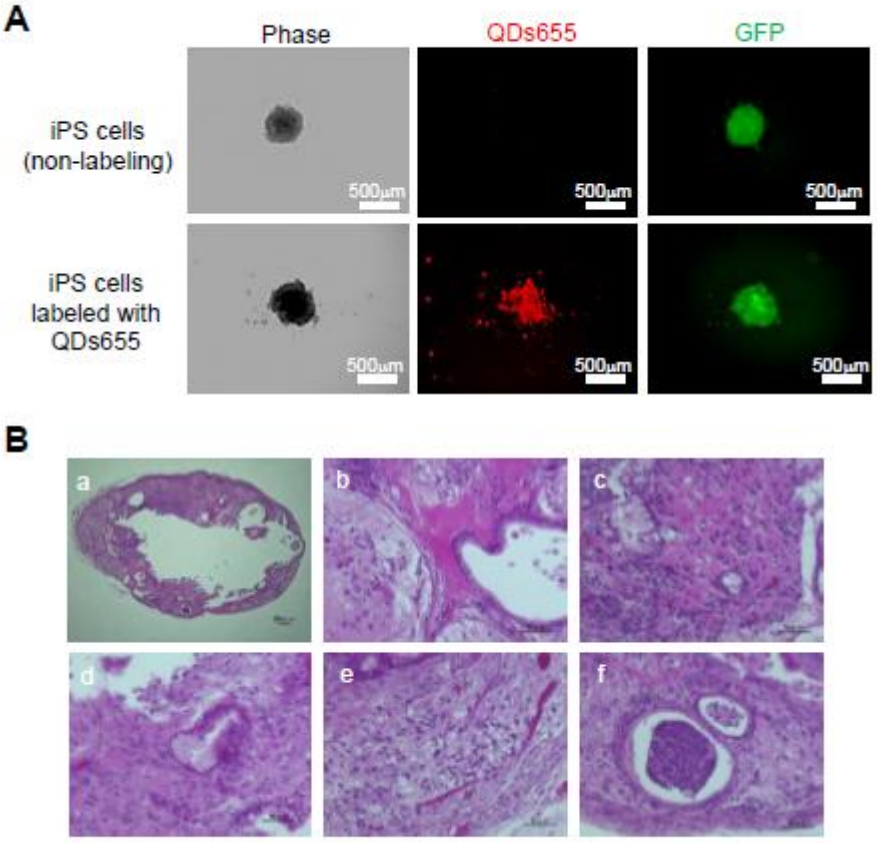


Figure 5



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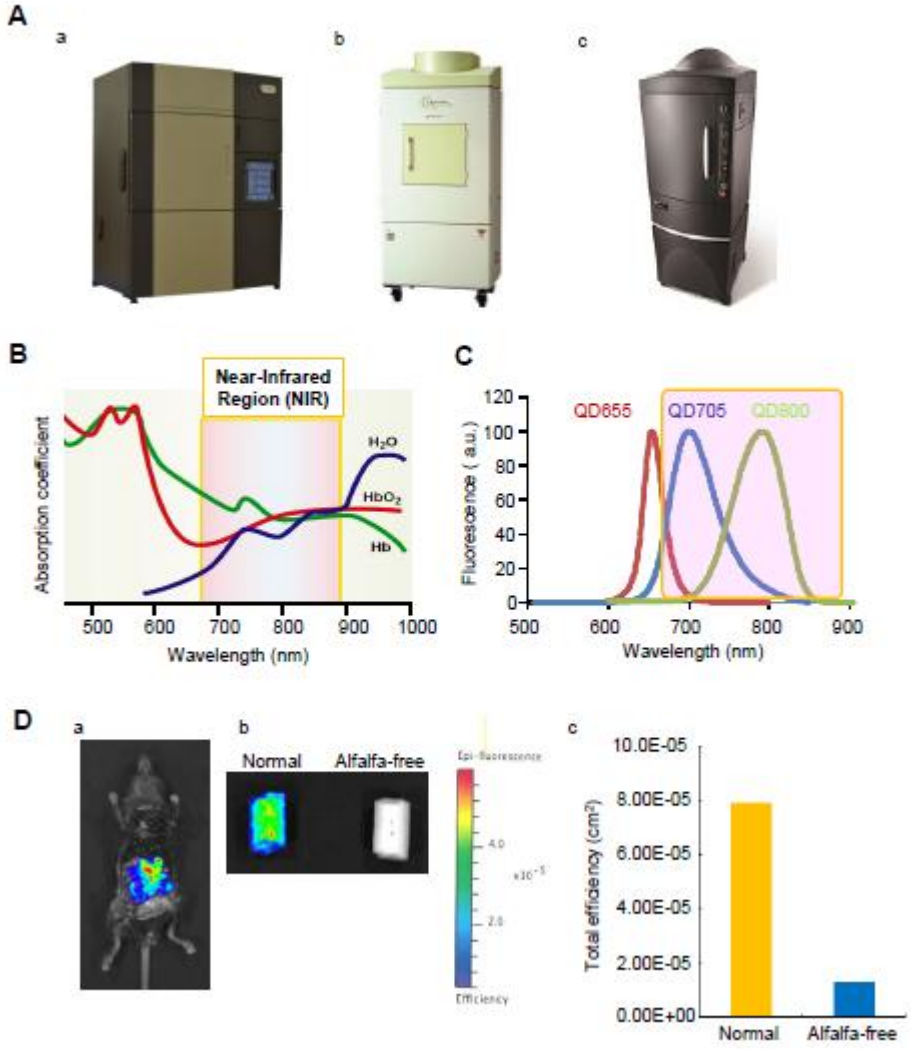

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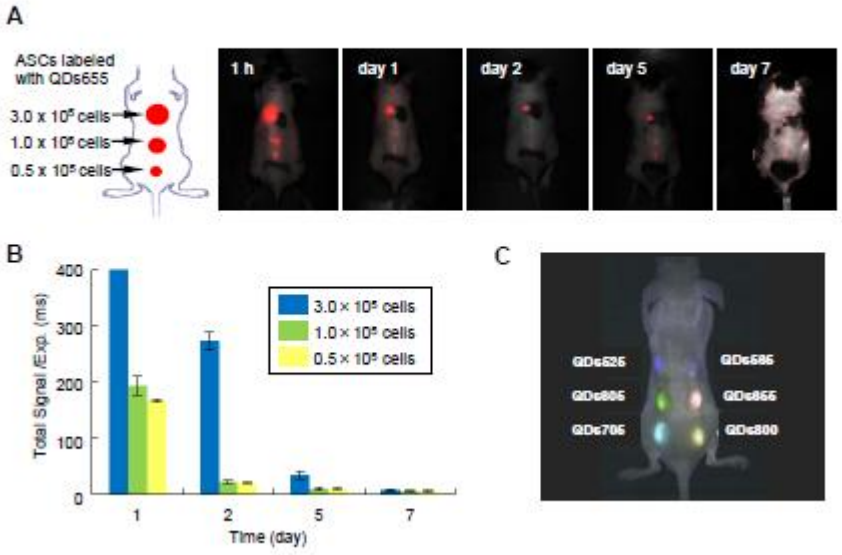

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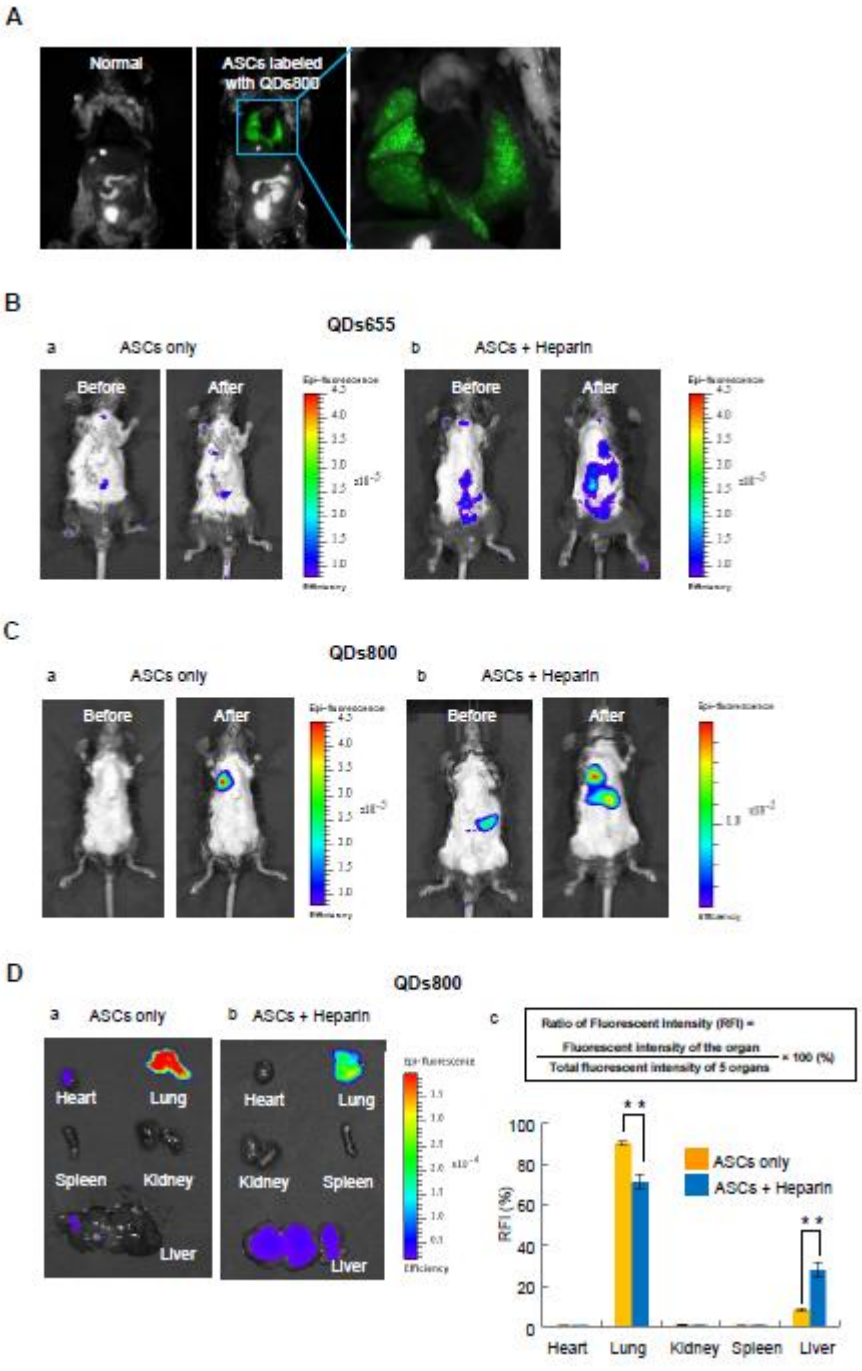
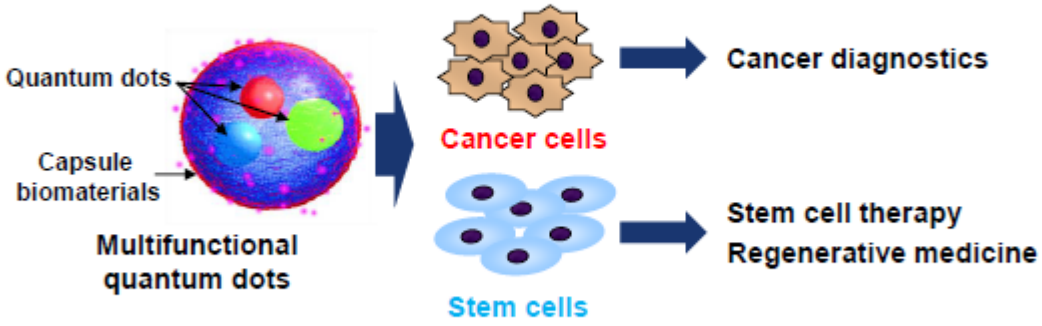


Figure 9



Graphical abstract