Self-illuminating quantum dots for non-invasive bioluminescence imaging of mammalian gametes

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Abstract

Background: The fertility performance of animals is still a mystery and the full comprehension of mammalian gametes maturation and early embryonic development remains to be elucidated. The recent development in nanotechnology offers a new opportunity for real-time study of reproductive cells in their physiological environments. As a first step toward that goal, we evaluated the effectiveness of a fluorescent and luminescent nanoparticle for in vitro and ex vivo imaging of porcine gametes.

Methods: Freshly harvested boar sperm were labeled with red-shifted (655 nm) quantum dot nanoparticles conjugated (QD+) or not (QD−) with plasminogen antibody and evaluated. Subsets of labeled spermatozoa were loaded into straws and placed within the lumen of gilt reproductive tracts for ex vivo intra-uterine imaging. Porcine cumulus-oocyte complexes (COCs) were matured in the presence of QD− or QD+. Ovarian follicles were microinjected with QD− or QD+ and placed in culture for up to 4 days. After labeling, all samples were supplemented with coelenterazine, the luciferase substrate, and immediately submitted to bioluminescence analysis, followed by fluorescence and hyperspectral imaging. Data were analyzed with ANOVA and P < 0.05 indicated significant differences.

Results: All labeled-samples revealed bioluminescence emission that was confirmed by fluorescence and hyperspectral imaging of the QD localization within the cells and tissues. Over 76% of spermatozoa and both immature and mature COCs were successfully labeled with QD− or QD+. The QD− fluorescence appeared homogenously distributed in the oocytes, while found in the entire sperm length with a higher accumulation within the mid-piece. Labeled-follicles exhibited a progressive migration of QD nanoparticles within the follicle wall during culture. In contrast, QD+ fluorescence signals appeared condensed and stronger in the follicle cells, sperm head, and sub-plasma membrane area of mature oocytes. Weaker QD+ signals were detected in the cumulus cells. Fluorescence and hyperspectral microscope imaging showed comparable intracellular QD localization. Ex-vivo intra-uterine bioluminescence imaging of labeled spermatozoa revealed stronger signals captured over the oviducts, with uterine body allowing the lowest signal detection.

Conclusion: Findings indicate that conjugated and non-conjugated fluorescent nanoparticles can be used for effective labeling of mammalian gametes for in vitro monitoring and potential in vivo targeted-imaging.

Keywords: Bio-imaging, Bio-sensing, Follicle, Nanoparticles, Oocyte, Plasminogen, Spermatozoa

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Background

The successful reproduction in mammals is orchestrated by successive and complex events occurring in tissues that are deeply embedded in the animal body. Thus, the examination of mammalian gametes and embryos has been mainly invasive; however, the recent progress in imaging systems, such as the digital videomicroscopy, allows for non-invasive and real-time investigation of gametes and embryos in their physiological conditions [1]. The combination of these imaging systems with fluorescent dyes has the potential to provide a better understanding of the biological and physiological processes related to successful reproduction. Few studies have attempted non-invasive imaging of spermatozoa [2, 3], using fiber-optic fluorescence imaging based upon the green fluorescence protein that is known to have tremendous brightness and photo-stability limitations for deep-tissue imaging. These challenges can be overcome with fluorescent inorganic nanoparticles [4]. The recent progress in nanotechnology provides a new horizon to unfold the mystery of the multifaceted molecular networks that are associated with oocyte maturation and sperm function [5, 6].

Inorganic semi-conductor quantum dots are size-tunable particles of up to 10 nm in diameter. Their unique optical and electronic properties allow size-dependent emission of photo-stable and bright fluorescence, from ultraviolet to near infra-red [7–10]. Over the past two decades, the specific features of quantum dots have been exploited for bio-imaging in biomedical research, especially by rendering them compatible with biological fluids [11–14]. Thus, the possibility to cross-linking these nanoparticles to various biomolecules (protein, antibody, peptide, DNA, etc.) has made them very attractive tools for non-invasive and real-time bio-imaging through cell labeling, single molecule or cell tracking, and diagnostic and targeted therapy [8, 15–17]. The attachment of proteins such as luciferase makes quantum dots suitable for both bioluminescence and fluorescence imaging [6, 18, 19], with the bioluminescence component serving for routine and rapid laboratory confirmation of labeling and the fluorescence part for deep tissue imaging through specific targets, such as protein [20].

Recently, we used luciferase-conjugated fluorescent quantum dots (BRET-QD) to assess their interactions with boar spermatozoa [5]. The QD-BRET complex is a self-illuminating nanoparticle that emits light under incubation with coelenterazine, the luciferase substrate [6]. This enzymatic reaction generates enough energy to excite the quantum dot core, leading to an intense red-shifted fluorescence emission (655 nm) that is crucial for deep-tissue molecular imaging [21]. Our previous study using this nanoparticle complex was an attempt to build on this dual imaging technology to enhance our comprehension of the complex biological and physiological processes of reproduction through non-invasive and real-time analyses [5]. In that pioneer study, we found that, when properly used, BRET-QD nanoparticles interact with spermatozoa without impairing their motility, quality (integrity of plasma and mitochondrial membranes), and viability (ability to fertilize the oocytes) characteristics.

In the present study, we expanded the potential use of QD-BRET nanoparticles for non-invasive imaging of spermatozoa within the female genital tract, while exploring the possibility for imaging cultured ovarian follicles and in vitro matured oocytes. In parallel, we conducted a targeted bio-imaging using the QD-BRET tagged with anti-porcine plasminogen antibody. In previous studies, this protein has been detected in the oocyte and has putative role during fertilization [22–24].

Methods

Materials and reagents

Stock solutions of coelenterazine (0.5 mg/ml methanol) and CdSe/ZnS core-shell structure quantum dots (500 nM in Tris buffer) cross-linked to Renilla luciferase (BRET) and nona-arginine R9 peptide (QD-BRET) were purchased from Zymera Inc. (San Jose, CA, USA). Functionalized QD-BRET complexes with the anti-porcine plasminogen antibody (PLG; cat#BP750, Acris Antibodies, Inc., San Diego, CA, USA) were prepared and purchased from Zymera. The functionalization did not affect the QD-BRET fluorescence and both functionalized (QD+) and non-functionalized (QD−) nanoparticles were used in the study. Additional, the anti-human plasminogen antibody was purchased from Santa Cruz Biotechnology (cat#25546; Santa Clara, CA, USA) for confirmation of the functionalized-QD-BRET labeling. Boar semen was purchased at a commercial boar stud (Prestige Farms; West Point, MS, USA) and reproductive tract with pre-ovulatory ovaries were collected from post-mortem gilts. Fresh samples were transported to the laboratory for the purification of living spermatozoa or the collection of follicles and COCs. Otherwise indicated, all other reagents were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Washing medium consisted of a pre-warmed PBS supplemented with 1 mg/ml of PVP.

Evaluation of synthesized QD-BRET nanoparticles

Three options were chosen to evaluate both functionalized (QD+) and non-functionalized nanoparticles. Aliquots of both QD-BRET nanoparticles were prepared for (1) transmission electron microscope or TEM imaging (Jeol 2100 Lab6 200 kV TEM operated at 200 kV), using a standard protocol; (2) dynamic light scattering (DLS;
and all experiments were repeated four times with independence.

The presence of ionophore served as a positive control to induce acrosome reaction. All samples were subjected to a flow cytometry analysis of the QD labeling and FITC-PSA staining. The flow cytometer (Becton–Dickinson FACSDiva version 6.1.3) was equipped with a quantum dot 655 nm filter and a 488 nm argon laser excitation. The proportions of sperm labeling in each factorial arrangement and controls (samples with or without ionophore) were evaluated. Experiments were repeated three times with independent sample collections and a minimum of 3,000 sperm cells were analyzed per sample in each experimental replicate.

**Tissue preparation and labeling**

**Ovarian follicles** A protocol developed by Wu et al. was used with a minor modification in this study [26]. Briefly, healthy antral follicles (4–8 mm in diameter) were dissected from pig ovaries in North Carolina State University-23 (NCSU-23) holding medium supplemented with 3% BSA-Fraction V (mg/ml). Dissected and trimmed follicles were placed in the culture medium consisting of NCSU-23 medium supplemented with 3 mg/ml BSA, 1% (v/v) Insulin-Transferrin-Selenium (ITS), 1.5 ng/ml porcine follicle-Stimulating hormone (pFSH), 30 ng/ml human Luteinizing hormone (hLH), 7.5% (v/v) porcine serum, and 1% (v/v) Penicillin/Streptomycin. The culture took place in 24-well tissue culture plates with 2–3 follicles per well containing 2–3 ml of culture medium. After 24 h of culture at 37°C under 5% CO₂, in a humidified environment, follicles were microinjected (Femtojet microinjection system; Eppendorf, Hauppauge, NY, USA) with 5 μl of PBS or 60 pmol (in 5 μl) QD1− or QD1+, and follicles were returned to culture (Day 0) for an additional 4 days (Day 4). Half of culture media was renewed every other day, from Day 0. Subset of follicles was not microinjected for later use as controls, for auto-fluorescence or -luminescence.

**Reproductive tracts** On the day of experiment, reproductive tracts of gilts were freshly collected at a local abattoir and transported to the laboratory on ice. All genital tracts were washed several times, cut into anatomic sections (uterine body, uterine horn, and oviduct), and all sections were kept in the washing medium until use for imaging. One tract was used for each replicate and a total of three independent replicates were performed.

**Bioluminescence imaging**

After labeling, all groups of spermatozoa (10⁶/0.5 ml of labeling medium) were washed three times by centrifugation (1,000×g, 3 min) to remove the excess of nanoparticles. Sperm pellets were resuspended in 50 μl of washing medium and kept into 1.5-ml Eppendorf tubes for in situ imaging. For ex vivo intra-uterine imaging, labeled (QD-) and unlabeled (QD0 or control) spermatozoa were
separately loaded into 0.5-ml plastic straws that were subsequently placed outside and inside the reproductive tract sections for imaging. Groups of approximately 50 labeled and unlabeled COCs were manipulated separately and washed three times by successive steps, followed by their transferred into a 96-well black bottom plate containing 50 µl of PBS for imaging. Ovarian follicles were collected on Day 1, Day 2, and Day 4 post-nanoparticles microinjection.

For bio-imaging, sperm suspensions and COCs were respectively supplemented with 4 and 2 µg of coelenterazine, while follicles were microinjected with 750 ng. All samples were immediately imaged (within 5 min) for bioluminescence signal or photon emission using the IVIS 100 imaging system (Perkin Elmer, Hopkinton, MA, USA), as previously reported [5]. The total photon emission data were recorded as photons/s. However, the proportion of light transmitted and captured across the surface of genital tract tissue sections was calculated as the total photon emission of luminal labeled spermatozoa (Inside) divided by the total photon emission of labeled spermatozoa outside of the genital tract ([Inside/Outside] × 100).

Fluorescence, hyperspectral, and transmission electron microscope imaging

After bioluminescence imaging, all samples were kept for in situ fluorescence imaging of quantum dots within the cells. Labeled and non-labeled samples were fixed in 4% methanol-free paraformaldehyde (spermatozoa and COCs) or 10% formalin (follicles) solutions. Fixed follicles were submitted to histological preparation, and sections of 4–6 µm thickness were generated. All other samples were smeared or placed on histology slides, rinsed, and mounted with appropriate medium for imaging.

Laser fluorescence imaging was performed with the confocal microscope (LSM510, Carl Zeiss Micro Imaging GmbH, Jena, Germany). For direct imaging, anti-human plasminogen antibody (Santa Cruz Biotechnology) was used for in situ immunofluorescence detection using FITC-conjugated secondary antibody as previously described [27]. After mounting on microscope histology slides with medium containing DAPI to counterstain nuclei, slides were used for confocal fluorescence imaging. Microscope filter sets of 420/40 Excitation, 660/40 Emission, and 475 DCXR dichroic were used and the background fluorescence of samples without nanoparticles was performed with the CytoViva imaging technology (CytoViva Inc.; Auburn, AL, USA). Spatial and spectral data were collected in each pixel of spermatozoa, COCs, and follicle sections that were fixed on histology glass slides. 

Hyperspectral data were quantified (CytoViva Hyperspectral Image Analysis Software algorithm). PBS-dispersed quantum dot nanoparticles were used to create the reference spectral library by comparing the particle filter results to a negative control sample, which removes any false positive spectral data and ensures valid results. Dark-field optical images of samples were taken and scanned with the reference spectral library to match pixels corresponding to the nanoparticles. Matching pixels were mapped in a pseudo red color to illustrate the presence and location of the nanoparticles in cells.

Transmission electron microscopy was performed on spermatozoa only. Sperm samples previously labeled with functionalized (QD+) or non-functionalized (QD−) QD-BRET were prepared for TEM as previously described [5]. Images were taken with the Jeol 2100 Lab6 200 kV TEM, operated at 200 kV.

Statistical analyses

Analyses were performed with the IBM SPSS 22.0 software package. One-way-ANOVA was used to test the effect of the nanoparticle labeling (fixed factor) on all dependent factors (bioluminescence and fluorescence intensities and viability data). When statistical differences were observed p < 0.05, analyses were followed by pairwise comparisons (Fisher’s LSD post hoc test). All data are expressed as mean ± SEM, unless otherwise indicated.

Results and discussion

Numerous challenges associated with deep-tissue characteristics drastically limit the broad application of bio-imaging technologies in large animals [28]. Especially in the reproduction field, the need of high spatio-temporal resolution and satisfactory contrast imaging technologies to detect spermatozoa and perform molecular or functional analyses are essential to overcome these challenges [1, 29]. Here we tested the interaction of QD-BRET (QD−) nanoparticles with pig gametes and ovarian follicles, for non-targeted and targeted bio-imaging in in vitro (labeled-spermatozoa and -oocytes), ex vivo (intraterine labeled-spermatozoa), and in situ (labeled-follicles during culture) settings. Previous studies have shown the non-toxicity of the QD− nanoparticles on boar spermatozoa [5] and other somatic cells [14, 16, 20, 30], when used at appropriate concentrations. For targeted imaging, we used QD− nanoparticles that were tagged with anti-porcine plasminogen antibody (QD+).

Plasminogen is an inactive zymogen mainly synthesized by the liver and at lesser extent in other tissues such as testes [31, 32]. Its secretions are found in various extracellular fluids, including seminal plasma and oviductal fluids [33, 34]. Plasminogen can specifically bind
to both gametes and its conversion into the serine protease plasmin by sperm-bound urokinase-type plasminogen activators (u-PAs) contribute to the regulation of fertilization [22, 23, 34, 35].

**Evaluation of synthesized QD-BRET nanoparticles**

All data are summarized in Figure 1. The calculated average (±SD) of random measurements of QD core–shell (CdSe/ZnS) diameters through the transmission electron microscopy (TEM) images was 8.2 ± 1.7 nm, which appeared little bit higher than our previous report [5] using a different batch of QD-BRET nanoparticles prepared by the same company (Zymera, Inc.). The tediousness of measuring quantum dot core–shells on the TEM may likely explain variations observed between studies. Both QD− and QD+ stocks were dispersed in the PBS (pH 7.4) solution used to label spermatozoa, for hydrodynamic dynamic light scattering size measurements. Analyses clearly showed functionalized (QD+) and non-functionalized nanoparticles with significantly different diameter sizes by intensity (32 ± 1.3 vs. 26 ± 1.3 nm, respectively; P < 0.05—t test) or by volume (40.4 ± 1.8 vs. 32.2 ± 0.9 nm, respectively; P < 0.05—t test). Both solutions showed presence of aggregates that appeared larger in QD− (239 ± 8.6 nm) than QD+ (153 ± 3.7 nm) samples (Additional file 1: Figure S1). The use of undispersed samples straight from the stocks to agarose gel electrophoreses confirmed the size difference of both nanoparticles. The functionalized nanoparticles (QD+) were heavier and slower to migrate through the gel than their non-functionalized counterparts (Figure 1), although we cannot tell whether the gel imaging is showing aggregate or non-aggregate sizes. The technical approaches used and results obtained with non-functionalized nanoparticles are consistent with a previous study verifying the conjugation of luciferase (Luc8) to quantum dot emitting at 655 nm [6].

**Gamete labeling and bioluminescence imaging**

Spermatozoa incubated and cumulus-oocyte complexes (COCs) matured in the presence of 0, 0.1, and 1 nM QD− showed dose-dependent like production of bioluminescence (light) emission (Figures 2, 3). In comparison

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**Figure 1** Characterization of the designed QD-BRET nanoparticles. Nanoparticles were analyzed with transmission electron microscopy (TEM), dynamic light scattering (DLS), and agarose gel electrophoresis. The TEM image shows the core–shell of the quantum dot nanocrystals averaging 8.2 ± 1.7 nm (SD, of 20 measurements); while the DLS measured the diameter sizes (n = 5) of the functionalized (32 ± 1.3 nm) vs. non-functionalized (26 ± 1.3 nm) QD-BRET that were significantly different (a, b; P < 0.05; T test). The agarose gel shows differential migration of both nanoparticles, with functionalized aliquots (1+, 2+, and 5+) being heavier and migrating slowly than their non-functionalized counterparts (1−, 2−, and 5−). The gel was loaded with various concentrations of nanoparticles (1, 2, and 5 nM/well, equivalent to 3 × 10^11, 6 × 10^11, and 15 × 10^11 nanoparticles, respectively) A 100 base pair (bp) PCR DNA ladder (Lad.) and sperm genomic DNA (gDNA) were loaded alongside for quality control of the gel electrophoresis.
Figure 2. Bioluminescence imaging (BLI) of spermatozoa. A) BLI of spermatozoa labeled with 0 nM (Control), 0.1 nM (QD0.1−), and 1 nM (QD1−) QD-BRET. Spermatozoa labeled with QD conjugated with anti-plasminogen antibody (QD1+) are also shown. Corresponding supernatants containing excess of QD− or QD+ are also imaged (bottom tubes, hand-marked S1). All samples were mixed with coelenterazine, the luciferase substrate before BLI. The quantification of total signals (photons/s) is summarized in B. Data are mean ± SEM of four independent replicates, and different letters indicate significant difference between columns (P < 0.05; ANOVA 1).

Figure 3. Bioluminescence imaging (BLI) of cumulus oocyte complexes (COCs). A) BLI of COCs matured in the presence of 0 nM (Control), 0.1 nM (QD0.1−), and 1 nM QD-BRET and 1 nM QD-BRET conjugated with anti-plasminogen antibody (QD1+). All samples were mixed with coelenterazine, the luciferase substrate, before BLI. The quantification of total signals (photons/s) is summarized in B. Imaging of each experimental replicate a included well without COCs, but filled with comparable volume of PBS as in the experimental groups to allow the evaluation of the PBS autofluorescence contribution to the results. Data are mean ± SEM of four independent replicates. Columns with different letters are significantly different (P < 0.05; ANOVA 1).
to their respective controls, the total fluxes (photons/s) were increased by 59× and 223×, in spermatozoa (Figure 2) and 1.6× and 1.7×, in COCs (Figure 3) by the presence of 0.1 and 1 nM QD− (P < 10⁻⁴; ANOVA 1). In contrast, lower and higher bioluminescence signals were respectively observed in spermatozoa and COCs labeled with 1 nM QD+ than their counterparts with 1 nM QD− (P < 0.05; ANOVA 1).

Data with spermatozoa are in agreement with our previous report [5], describing interaction of QD-BRET (QD−) nanoparticles with boar spermatozoa without affecting their function (motility and fertilization). Here we report for the first time the incorporation of QD− by porcine COCs, while their interactions with functionalyzed nanoparticles (QD+) indicate the presence of plasminogen protein in both gametes. This successful interaction were further confirmed through transmission electron (Additional file 2: Figure S2) and confocal laser (Additional file 3: Figure S3) microscopy analyses, showing higher accumulations of quantum dots in spermatozoa (TEM) that corresponded to similar area targeted by anti-plasminogen antibody (the sperm head). Overall and despite using different sources of antibodies (Acris and Santa Cruz), generated images appear alike, which position the use of nanoparticle-based imaging as a viable approach for non-invasive functional analyses of desired proteins, such as the plasminogen/plasmin system, having putative role during fertilization [22, 24]. The current direct and indirect (through nanoparticles) findings bring further confirmation of the presence of plasminogen on mammalian oocytes [23], while revealing its presence in spermatozoa that has not yet been clearly demonstrated in the literature.

**Ex vivo intra-uterine bioluminescence imaging of spermatozoa**

Accurate and non-invasive investigations of mammalian gametes in physiological conditions are limited with the conventional techniques and imaging approaches [36–39]. Consequently, the progression and behavior of gametes, especially spermatozoa within the female reproductive tract remains a mystery. As a first step toward to elucidating this physiological event, the current experiment was developed for a possible tracking of spermatozoa through the capture of luminal sperm-emitting photons over the reproductive tract surface.

Bioluminescence signals (photons/s) were captured above background on each reproductive tract section (Figure 4). A higher proportion of light was transmitted through the oviduct section (21.5 ± 2.4%), compared to the uterine horn (7.2 ± 0.9%) and body (1.1 ± 0.4%) (P < 0.01; ANOVA 1). The low proportions of transmitted lights underline the challenge of deep-tissue imaging, while indicating the possibility of using nanotechnology
approach for successful imaging of spermatozoa within the genital tract lumen. The current imaging was based upon the bioluminescence property of the QD-BRET, which brightness is till limited for deep tissue imaging. Yet, the red fluorescence capability of these nanoparticles remains to be exploited.

The inorganic nature of quantum dots (QD) offers a great potential for bright and photo-stable fluorescence signal to allow intra-uterine imaging of spermatozoa. However, the successful utilization of QD nanoparticles for the purpose-bio-imaging may fully rely on miniaturized devices, such as the probe-based confocal laser endomicroscope (pCLE). This instrument uses light in the visible spectrum that should be enough to excite the QD nanoparticle, and provide adequate spatial resolution by the mean of flexible miniaturized fiber optic probes of 1.5–2.6 mm outer diameter. The pCLE is being used for imaging diagnoses in the clinical area [40–42], and has been tested for ex vivo studies of spermatozoa in human testicle [3] and ewe oviduct [43] using organic fluorochromes.

Sperm labeling and viability evaluation through flow cytometry
Flow cytometry is an extensively used approach to study a variety of single sperm attributes with high precision, accuracy, and low costs using fluorescence techniques [44]. Proportions of 64 ± 4.5%, 76 ± 4%, and 91 ± 2% of spermatozoa were successfully labeled with 0.1 nM (QD0.1−), 1.0 nM (QD1−), and QD1+ compared to nothing in the control or QD0 group (P < 0.05). Surprisingly, the mean relative fluorescence intensities (RFI) of QD1− and QD1+ remained comparable, but were both significantly higher than those in the control and QD0.1− (Figure 5A; P > 0.05). We attributed this discrepancy to the well-known brightness fluorescence of quantum dots, while the co-functionalization of QD-BRET with luciferase (Luc8) and antibody may limit the amount of Luc8 per QD, leading to low bioluminescence emission. Nevertheless, the findings suggest that most spermatozoa express plasminogen protein, and the limited-expression on the sperm surface, mostly in the head area, may result in a low overall bioluminescence emission.

As for the viability of labeled spermatozoa, the intactness of their acrosome membrane was evaluated. The control group showed a proportion of 86% of spermatozoa with intact acrosome, while 85% of spermatozoa labeled with QD1+ versus 71% with QD1− maintained intact acrosome membrane (Figure 5B; P < 0.05). This finding indicates the possibility of small-sized QD-BRET (20–25 nm) to damage the sperm acrosome membrane. In possible support of this speculation, a recent study has reported the internalization of small-sized gold nanoparticles (less than 10 nm) and the exclusive localization of larger nanoparticles on the plasma membranes of intact bovine spermatozoa [45]. In the other hand, the absence or reduced negative effects in the QD1+ group may be attributable to the bigger size of functionalized nanoparticles (32 vs. 26 nm to QD1−) and more likely to their specific interactions with plasminogen proteins found on the sperm surface membrane. Furthermore, the formation of nanoparticle aggregates are another potential sources of toxicity to cells, including spermatozoa. Barchanski et al. have detected higher accumulations of conjugated-gold nanoparticles in the post-equatorial region of acrosome-reacted spermatozoa, which commend for further and close investigations of quantum dot interactions with spermatozoa [45]. These investigations will contribute to further optimize the use of quantum dots for non-invasive imaging of fully functional and viable spermatozoa. Great interests are expected from controlled-uses of nanoparticles for sperm labeling in assisted reproduction. Here we used a total of 3 × 1011 nanoparticles to label 100 million spermatozoa, corresponding to 3,000 nanoparticles per sperm cell that is well-below the 14,000 gold nanoparticles inducing detrimental effects on bovine spermatozoa [46]. The current findings complement a previous report indicating no
major losses of motility, plasma and mitochondrial membrane integrities, and fertilizing potential of pig spermatozoa labeled with 1 nM QD (or QD1−), equivalent to 3,000 nanoparticles/sperm cell [5].

**Ovarian follicle labeling and bioluminescence imaging**

The ovarian follicle growth is concomitant with the oocyte development and the formation of an intra-follicular cavity surrounded with various cell types. This cavity is filled with a fluid resulting from blood exudates and other follicular cell secretions, which dynamic concentrations in molecules such as hormones and growth factors regulate the growth of both follicle and oocyte [47, 48].

Here, the microinjection of QD nanoparticles within the follicular cavity constituted an attempt to develop a strategy for intra-follicular imaging for biosensing and tracking of key molecules that intra-follicularly influences oocyte quality and viability. Figure 6 shows bioluminescence imaging of QD-BRET nanoparticles with (QD1+) or without (QD1−) the plasminogen antibody within the ovarian follicles. Follicles microinjected with QD1+ always displayed greater luminescent signals than those with QD1− (Figure 6; Line B in Red box vs. White box and Line E vs. Line D). No signals were observed in the control groups, consisting of follicles microinjected with either PBS (CTL, Line A), coelenterazine (CTL, Line B), or QD1− without coelenterazine (Line C). Interestingly, the microinjection of QD1− or QD1+ did not cross-contaminate the neighboring follicle (Figure 6; Lines D, E). This observation is important as ovarian follicles are independent structures with different health and developmental status, leading to the production of oocytes with different developmental competences. Follicles microinjected with QD0.1− or QD1− exhibited dose-dependent like light emission at all time-points (Day 1, Day 2, and Day 4—P < 0.05; Figure 7). Interestingly, follicles microinjected with QD1+ maintained the highest light emission throughout the culture period (P < 0.05; ANOVA-1).

Although the viability of cells was not verified, data suggest that self-illuminating luminescent nanoparticles can be used for molecular imaging of cultured follicles. Our finding adds to a recent study reporting a successful intra-follicular imaging through a transgene carrying the luciferase reporter gene [49]. The current work paves the way for further optimization of this novel non-invasive imaging approach that will lead to promising in situ real-time description of the oocyte maturation and related intra-follicular key molecules.

![Figure 6](image_url)

**Figure 6** Bioluminescence imaging of QD-BRET (QD) microinjected cultured ovarian follicles. Follicles were microinjected with QD1− or QD1+ during in vitro culture. Imaging was performed with follicle controls, microinjected with only PBS (Line A, CTL), QD1− (Line A, White Box), or QD1+ (Line A, Red Box), follicles microinjected with both coelenterazine and QD1− (Line B, White Box), or QD1+ (Line B, Red Box). The inset shows follicles microinjected with coelenterazine only (Line C) or with QD1− and QD1+, showing respective bioluminescence emissions in Lines D and E.
Confocal fluorescence microscope imaging of the nanoparticle bio-distribution

Following incubation with QD1−, the fluorescence signal appeared more diffuse on the entire spermatozoon, with the strongest signal being observed in the mid-piece section (Figure 8). This cellular distribution contrasted with the QD1+ labeling that was mainly limited to the sperm head (Figure 8). This observation brings a direct evidence of the plasminogen protein presence in boar spermatoza. The spatial distribution of QD1− and QD1+ fluorescence signals may contribute to explain the differential bioluminescence emission between both groups.

On the other hand, the QD1− fluorescence signal appeared homogenously distributed within the oocyte and cumulus cells, before (Figure 9c, d) and after maturation (Figure 9e, f). However, this signal appeared more organized in oocytes matured in presence of QD1+, with lesser or no signal being detected in the cumulus cells (Figure 9g, h). Interestingly, the invasive detection
through antibody revealed a strong accumulation of plasminogen in mature versus immature oocytes (Additional file 3: Figure S3). Both nanoparticle-based (non-invasive) and direct (invasive) detection of plasminogen showed comparable distribution within the matured oocyte.

The bio-distribution of QD1− within the follicle appeared dynamic during culture. A great accumulation of the QD fluorescence signal was seen on the mural granulosa cells that surround the follicular cavity on Day 1 post microinjection. The signal appeared within the theca internal on Day 2 and then reaches the farthest layer of cells, the theca external, on Day 4. Interestingly, microinjected QD were able to penetrate the cumulus-oocyte complex, with a higher accumulation found in the oocyte (Figure 10).

Overall, the fluorescence imaging brings further confirmation of the bioluminescence observations and provides direct evidence of the plasminogen protein presence in pig gametes and ovarian follicles. Thus, nanotechnology approach based on self-illuminating quantum dots could be used as a novel methodology to measure the dynamic changes in molecular events occurring during intra-follicular growth and maturation of the oocyte. In this study, it is likely that the combination of QD-BRET ± antibody complexes with the cell-penetrating peptide, nona arginine R9, facilitates their incorporation into follicular and cumulus cells, which likely transfer incorporated-nanoparticles to the oocyte through gap-junctions uniting both cell types (cumulus cells and oocytes). The conjugation of nanoparticles with cell penetrating-peptides (CPP) has been shown effective for interactions with cells, including mammalian gametes despite different depth of penetration efficiencies between CPP [45]. Our findings using a polycationic CPP coupled to quantum dots are in agreement with a recent work showing accumulation of BSA-coated gold nanoparticles in porcine oocytes and surrounding cumulus cells [50]. These authors found no detrimental effects of gold nanoparticles on pig oocytes and spermatozoa, which is in line with our present and prior findings using quantum dots [5]. It is important to mention that these results were obtained with comparable nanoparticle diameter sizes (20 vs. 26 ± 1.3 nm for gold and quantum dot nanoparticles, respectively) and numbers.
(1.24 \times 10^{10}/COC and 1.23 \times 10^{3}/sperm cell for gold vs. 3 \times 10^{11} and 3 \times 10^{5} for quantum dot nanoparticles, respectively). Yet, numerous studies have evidenced that the penetration of nanoparticles into spermatozoa remains limited or prevented by the composition and status of the plasma and acrosome membranes [5, 46, 51].

**Hyperspectral fluorescence imaging of the nanoparticle bio-distribution**

This novel nano-scale imaging technology was used to further confirm the QD labeling of cells and map their localization. The hyperspectral fluorescence imaging combines digital image capturing and conventional spectroscopy to generate spectral signatures as a function of wavelength that characterizes samples. Samples without labeling (negative control) showed no fluorescence signals (Figures 11a, 12a, d), while small aliquots of QD suspensions and samples labeled with QD revealed reference spectral libraries with peak emission of or near 655 nm (Figure 11b), corresponding to the emission wavelength of the QD used in this study.

The fluorescence signal of QD1− was mostly detected in the sperm mid-piece while those from QD1+ were stronger and essentially located in the sperm head (Figure 11d, f). These results corroborated with those of the confocal microscope. Similar fluorescence intensities were observed with the cumulus-oocyte complexes or COCs (Figure 12b, c) and follicles (Figure 12e, f). In both sample types, the QD1+ fluorescence remained stronger than that of QD1−. The fluorescence signal ratios (QD1+/QD1−) were increased to approximately 1.6×, in spermatozoa (Figure 11f, d) and 9.3×, in COCs (Figure 12b, c).

Both COCs and follicle hyperspectral fluorescence image intensities matched the bioluminescence data. The
application of this novel hyperspectral imaging validates the traditional microscope and confocal fluorescence technologies. The current study represents, to the best of our knowledge, the first report on mammalian reproductive cells that combines both nanotechnology and hyperspectral fluorescence for bio-imaging. The application of the hyperspectral fluorescence imaging is new in the reproductive field, but this technology has been applied in various research fields of biomedicine [52], including cancer and Parkinson's disease diagnoses [53, 54] and tissue characterization [55].

Conclusions
The current study uses various settings (in vitro, in situ, and ex vivo) to assess the effectiveness of a new nanotechnology-based imaging approach for mammalian reproductive cells. We combined traditional and novel imaging techniques to validate the nano-based labeling. The proposed-imaging technology offers the possibility for minimal invasive tracking of reproductive cells in their physiological environments, with the possibility to measure changes in cellular and molecular events that affect mammalian gamete quality. However, nanoparticle uptake by ovarian follicular cells and subsequent bio-imaging still need optimization in further experiments. Furthermore, the study confirms the presence of plasminogen protein in pig oocytes, while revealing its detection in mature spermatozoa. This immunopositive reactivity allows for further noninvasive and functional analyses of the influential

![Figure 11](image)

Figure 11 Hyperspectral fluorescence imaging of QD-BRET (QD) labeled spermatozoa. Negative control spermatozoa (a) were used to remove false positive signals and validation of QD-BRET fluorescence spectral libraries that were captured around or near 655 nm emission wavelength, as indicated by the arrow (b). Hyperspectral images of spermatozoa were taken (c, d, e) followed by the comparison of all pixels with the QD1− and QD1+ spectral libraries (Not shown here). Pixels that matched the spectral are mapped with a pseudo-red color (arrows) illustrating the presence and location of QD1− (d) or QD1+ (f).
range of plasminogen during the fertilization success and outcomes.

**Additional files**

**Additional file 1: Figure S1.** Dynamic Light Scattering (DSL) size measurements. Self-illuminating bioluminescent resonance electronic transfer quantum dots emitting at 655 nm (BRET-QD) and linked to nona-Arginine cell-penetrating peptides (R9) were functionalized (QD+) or not (QD-) with plasminogen antibody. Aliquots of each sample were dispersed in the incubation medium (PBS, pH 7.4) and submitted to the zetaPALS operated at 659 nm wavelength for DSL measurements at 37°C after 5 min equilibration. Monomer and aggregate sizes are indicated in black and blue colors, respectively.

**Additional file 2: Figure S2.** Transmission Electron Microscope imaging of labeled spermatozoa. Cross-section heads of spermatozoa labeled with functionalized (blue frame) and non-functionalized (black frame) QD-BRET are shown. Arrows indicate the localization of quantum dot (QD) core and higher accumulation can be seen in functionalized, compared to non-functionalized sections.

**Additional file 3: Figure S3.** Laser confocal microscope imaging of labeled cumulus-oocyte complexes and spermatozoa. The black frame regroups cumulus-oocyte complexes labeled with anti-human plasminogen antibody before (B) and after (C) in vitro maturation, while blue frame indicates spermatozoa labeled with the same antibody (E) and counterstained with DAPI for nuclei visualization (F). Micrographs A and D correspond to samples that were incubated without anti-plasminogen and served as negative controls. Arrow and arrow heads indicate the oocyte and the cumulus cells, respectively.

**Abbreviations**

FITC-PSA: fluorescein isocyanate conjugate-Pisum sativum agglutinin (pea);
BSA: bovine serum albumin;
PBS: phosphate buffered solution.

**Authors’ contributions**

JMF conceived and designed the study, performed most of the experiments, drafted and wrote the manuscript; JMG contributed to the experimental design, participated to the bioluminescence imaging and help drafting the manuscript; RCY contributed to the experimental design and participated to the bioluminescence imaging and analysis; PLR and STW participated to the experimental design and provided guidance; Authors have discussed the results and reviewed the manuscript. All authors read and approved the final manuscript.
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