1. Introduction

The field of nanotechnology is rapidly growing and it is estimated that there will be USD $12 trillion invested in nanotechnology globally by 2012 [1]. Nanoparticles (NPs) (particles of diameter 10–500 nm) are already used in the cosmetics industry and are being developed for drug delivery, diagnostic imaging and tissue engineering, to name but a few applications. The increasing number of NP applications also results in increased environmental and human exposure, and thus there is a need to investigate their potential detrimental effects. A global research effort is currently underway and focused on these so called Environmental, Health and Safety (EHS) concerns [2,3].

Currently, there is an international effort headed by organizations such as the OECD (Organization for Economic Co-operation and Development, e.g. Working Party on Manufactured Nanomaterials or WPNM) to develop research endpoints that will enable individual member states to implement sound, science-based regulatory frameworks for the burgeoning nanotechnology industry.

The inherent risk of NPs is the product of hazard and exposure. Hazard is the intrinsic toxicity of the NP, once in the organism. Exposure is the propensity of the NP to sequester in the organism (bioaccumulation), together with the likelihood of the organism encountering the NP. Important questions we seek to help answer are ‘If embryos are exposed to NPs, where will the NPs go? With what kinetics?’ and/or ‘Can we expect acute or chronic toxic responses?’ Furthermore, in the instance of accidental exposure, what response can be recommended? To help answer these questions, federal governments have called for studies to establish the relationship of nanomaterial risk with their physical chemistry.

There are as yet only a few systematic studies that relate NP properties to bioaccumulation in living organisms; and next to no nanotoxicity studies involving embryos have been conducted. Studies on the interactions of NPs with embryos are important since the problems associated with health of an adult organism could be due to exposure during the development process, and exposure may even affect embryonic viability. Indeed, there is a substantial knowledge gap with respect to NP risk assessment and policy/regulation development.

With the boom in NP development there is an increasing possibility of environmental and/or occupational exposure. A recent study has shown that NP in aquatic systems can work their way up the food chain via ingestion [4]. It is clear that potential risk needs to be evaluated, particularly by regulatory agencies. The most likely path of NPs into embryonic organisms is through their parents. For mammals this would result from crossing the placental barrier, for avian and aquatic vertebrate species this would occur in egg generation. Young organisms could be exposed through food ingestion or through their skin. Once inside an immature organism, NPs can trigger toxicity through a variety of pathways, but the most common are: reactive oxygen generation, membrane disruption, release of toxic components and immunological responses [5,6].

A significant difference between embryonic and mature organisms is that embryos undergo a large degree of new blood...
vessel growth (angiogenesis). These blood vessels tend to be leaky, which reflects the fact that angiogenesis is a lengthy process involving initial formation of vessels followed by their maturation. Unlike mature blood vessels, endothelial cells in angiogenic vessels do not seal tightly against one another. The hydrodynamic pressure of the liquid inside the blood vessel and concentration gradients push particles through nano-perforations (or fenestrations) between cells and into the surrounding tissue, similar to the enhanced permeability and retention (EPR) effect [7]. This effect has also been observed and exploited for delivery of NPs to tumors. The size and surface charge/chemistry dependences of NP sequestration into angiogenic tissues are currently unknown. Such sequestered NPs could leach toxic substances such as Cd and/or Se and/or cause a deleterious immunologic response [6].

There is a growing consensus that important physical properties of NPs are related to their potential EHS risk. The properties are: size, shape, surface charge density, surface chemistry and degree of aggregation [4,5]. It is believed that understanding how these properties influence bioaccumulation and cytotoxicity will lead to a predictive model for gauging toxicity of NPs. It is therefore critical to develop methods to analyze these properties of NPs both in vitro and in vivo, since NP properties might be affected by their biological environments.

We have been employing fluorescence correlation spectroscopy (FCS) and fluorescence cross-correlation spectroscopy (FCCS) in the study of semi-conductor nanocrystals (aka quantum dots) to measure their solution dynamics and photophysics [8–10]. Recently, we have optimized an FCS set-up to measure the properties of fluorescent NPs in the blood stream of chicken embryos. Briefly, FCS analyzes fluctuations in fluorescence intensity from a well-defined volume of excited molecules, where the fluctuations result from a changing number of fluorescence emitters (fluorophores) within the volume [11–14]. Thus, any phenomenon that alters the number or diffusion behavior of fluorophores within the volume will induce a change in the time dependence of fluorescence intensity fluctuations around the average. These effects could arise from simple diffusion, flow, chemical reactivity, aggregation, or photochemistry, and the time scales of the fluctuations are related to the kinetic rates of the phenomena. In FCCS, a signal is only possible when the (two or more) labelled species are physically linked and therefore FCCS can be used to interrogate the equilibria between different associating NPs and between NPs and biomolecules (serum proteins) [9,15,16].

In this article, we will examine the most recent approaches to quantify the disposition of NPs in the blood streams of live organisms. We will elaborate on FCS methodology as an approach to perform real time measurements of concentrations, sizes and aggregation states of NPs in chicken embryos. We will also propose near and long term future directions for this field of study.

2. Current approaches

NP toxicity studies have vastly expanded in recent years owing to their increasing applications of NPs in biological systems. There are a number of reviews on NP toxicity, including interaction(s) with biological materials [5,16–18]. The vast majority of these studies have been performed on cultured tissue cells and several excellent reviews detail the trends in these [19]. Briefly, at low doses (<10 μg mL⁻¹), most NPs appear to have low to minimal cytotoxicity regardless of whether they are carbon-, metal- or semi-conductor-based, at least for acute exposure. For higher NP doses, almost all cells lines show some toxicity (usually scored as lower viability). The most compelling physicochemical NP attribute that can be related to cytotoxicity is surface charge, with toxicity increasing in the following way neutral < anionic < cationic. It is hypothesized that the cationic NPs interact more strongly with cell membranes, which tend to be negatively charged. Once internalized into cells, a common mechanism is that certain NPs generate reactive oxygen species (ROS), which damage the cellular machinery. However, this is not true for all NPs and further mechanistic studies are needed. Moreover, there is a general call for more in vivo (i.e. whole organism) studies to examine hazard (i.e. toxicity) and exposure (i.e. routes of bioaccumulation) that go beyond the typical cytotoxicity studies alluded to above. For the current article, we will focus on methods which track the fate of NP once they are in the blood stream.

Recent work indicates that once in the bloodstream of mature animals, renal clearance will occur only for NPs smaller than 5 nm in lateral dimension [4]. Additionally, since nanoparticles are proving to be effective delivery agents for directing therapeutic cargo to angiogenic tumor tissues, one can expect a similar accumulation of NPs in angiogenic tissues of embryos. Recently, trophic transfer of NPs (here, CdSe nanocrystals) between aquatic invertebrates was demonstrated by Holbrook et al. [4]. Thus, NPs can be ingested and transferred between species. A challenge in detecting NPs in organisms further up the food chain was also identified by Holbrook et al. as there is limited bioconcentration and therefore classic analytical Cd detection is non-trivial.

Several groups have studied semiconductor nanocrystals (i.e. quantum dots or qdots) in the blood stream of rodents. In one of the first studies, Larson et al. demonstrated that qdots could be used to image vasculature (using two-photon excitation) in dermis of mice [12]. Nie and co-workers developed functionalized qdots for tumor targeting in mice [20]. In none of these important studies was it possible to measure directly the blood concentration of the qdots or whether or not they were aggregated.

2.1. Quantum dots as canaries in a coal mine

We have developed FCS-based methodology to measure various aspects of NPs in the blood stream of chicken embryos. We submit that since qdots surfaces can be relatively easily functionalized, that they may serve as good test particles for in vivo bioaccumulation and toxicity studies. For FCS studies, they have the advantages of high particle brightness and large light absorption cross-sections both for linear and non-linear excitation. Below is a brief quantum dot background relevant to this article.

Functionalized qdots show promise, not just as research imaging tools but also as diagnostic fluorophores in healthcare applications. Qdots boast many advantageous properties including; size-tunable, narrow emission spectra; broad absorption spectra; capacity for surface functionalization, and high photo-stability compared to organic fluorophores. These attributes have made qdots invaluable tools in many applications. One particularly compelling application is in multiplexed diagnostics where their narrow emission spectra of qdots allow for many molecules to be labelled and measured at once [20]. Qdot sensors based on fluorescence resonance energy transfer (FRET) [21] or on surface enhancement/quenching have also been developed for a range of molecules [22]. Qdots have also been developed for binding assays with FCS [9,15,16]. The sensitivity of qdot hydrodynamics to environmental factors may be one barrier to their widespread use for these purposes.

The use of qdots for long-term imaging in research and diagnostics is promising. The long-term fluorescence stability of qdots means that they can be used to track malignant cell proliferation and can be used to specifically label cancerous tissue [23]. This may allow improved cancer therapies for more complete removal
of malignant tumors in vivo. The interaction of qdots with blood vessel tissue may be an important factor in perfecting diagnostics for widespread use. The potential dysfunction that results from qdot localization in blood vessel walls will need to be investigated to ensure long-term benefits of in vivo treatments and diagnostics involving qdots. Moreover, the mechanism by which qdots enter blood vessel tissue has the potential to be generalized to other nanoparticles with similar surface coatings, such as MRI contrast agent NPs.

Qdots are frequently administered by intravenous or intra-arterial injection during biological studies on animal models of various diseases. Since blood vessels are the first tissue with which nanoparticles interact under these conditions, it is important to know whether qdots will interact non-specifically with tissues, proteins and cells in blood vessels and how their interaction with the endothelial lining of the vascular system may influence their absorption, distribution, metabolism and excretion in vivo. Knowing the characteristics of qdots that facilitate long circulation time scales (hours) or experience less interaction with blood vessel tissue may aid in the design of qdots that are optimized for: vasculature visualization, targeting toward cancerous cells, targeting toward disease diagnostic markers and for a minimal invasiveness in future applications. Moreover, interaction of qdots with endothelial tissue in blood vessels may provide insights into a mechanism for qdot toxicity and perhaps even the toxicity of NPs in general.

NP toxicity studies have vastly expanded in recent years owing to the increasing applications for NPs in biological systems. There are a number of excellent reviews on nanoparticle toxicity including NP interaction with lung surfactant [24] and by various entry pathways [17]. For qdots in particular, it is difficult to compare many systems because the range of qdot cores, capping materials and functionalization is vast. Qdot toxic effects are also largely model dependent: the localization and degradation observed is often cell, media or model specific. Unfortunately, investigation into the toxic effects of qdots is typically not the primary aim of most studies, but rather a secondary observation that can be made. As a result the concentrations and routes of administration vary widely. Finally, the long-term effects of qdots in vivo are expensive to measure with animal models. Nevertheless, an excellent review by Hardman [5] discusses some of the in vitro and in vivo experiments that have been performed relating to qdot toxicity.

In general, the potential toxicity of qdots could manifest in four main ways. Firstly, the NP core can be degraded releasing toxic ions into the biological system. Secondly, the capping material (and polymer) can interfere with metabolic reactions. Thirdly, owing to NP size, the NPs may localize to certain cell or organ areas, influencing function. Finally, NPs at high concentrations may influence the osmotic balance of the living system. Some of the observed effects and toxicity mechanisms have been summarized [5]. The information gained from studies on qdots could be generalized to other NPs if the effect occurs by one of the latter three mechanisms. Qdots make effective tools to study these latter three toxicity paths since they can function as quantitative fluorescence tools, as well as being potentially toxic NPs.

2.2. The chicken chorioallantoic membrane (CAM) as blood vessel model system

The CAM is a highly vascularized, transparent extra-embryonic membrane that grows against the inner wall of the developing chicken egg. As a model for angiogenesis (newly forming blood vessels), the CAM of the chicken embryo has many advantages. The chicken embryo has a short gestation period and the embryo itself is very easily manipulated [25]. The CAM develops between days three and twelve of the gestation period, and undergoes rapid angiogenesis between days five and nine [25]. Intussusceptive (invaginations of vessels) capillary growth occurs between days eight and twelve and is followed by a period of vessel expansion [25]. The CAM aids the developing embryo in nutrient and waste exchange with the atmosphere, especially calcium uptake from the shell and electrolyte transport from the allantoic sac [26,27]. The CAM is formed from three layers of tissue, the chorionic epithelium, the mesoderm and the allantoic epithelium [25]. The chorionic epithelium lies proximal to the shell and is derived from the chorionic ectoderm, while the mesoderm is the center tissue and consists of a highly branched vasculature system that undergoes rapid angiogenesis during development. The allantoic epithelium lies distal to the shell and is derived from the allantoic ectoderm. As the CAM develops and surrounds the entire inner shell, the vasculature extends from the mesoderm into the chorionic epithelium for more efficient nutrient exchange. As mentioned previously, because embryos undergo angiogenesis, the leakiness of the developing blood vessels will likely lead to accumulation of NPs from the blood stream.

2.3. FCS measurements of nanoparticles in biological milieu: peeling back the onion

In the following subsections, we will outline the techniques used to measure the properties of NPs in biological environments. First, FCS will be described both from theoretical background and experimental set-up points of view. Then, we will detail the examination of the effects of blood sera and whole blood on the NPs. Next, we discuss the collection of FCS data in the CAM blood vessels, when blood flow is halted. Finally, we show the results of NP injection into blood vessels of the CAM where blood is flowing.

2.3.1. Brief tutorial on FCS

Excellent reviews for FCS can be found in a recent reference book [28]. Only the pertinent background will be presented here. Fluorescence correlation and cross-correlation spectroscopy is based on the analysis of temporal intensity fluctuations $\partial F(t)$ in fluorescence about the time averaged fluorescence intensity, $\langle F \rangle$, from emitters in a well-defined interrogation volume. These fluctuations can arise from either changes in the instantaneous concentrations of the NPs (diffusion, flow, chemical change) or from photophysical changes. In general, the normalized fluorescence correlation function is defined by [9,11]:

$$G_0(\tau) = \langle \partial F_i(t) \partial F_j(t + \tau) \rangle / \langle F_i \rangle \langle F_j \rangle$$  \hspace{1cm} (1)

When $i = j$, Eq. (1) represents the temporal autocorrelation of fluorescence intensity fluctuations. In our work we employ two-photon excitation (TPE) of NPs. Autocorrelation decays were modeled assuming a Gaussian TPE volume using the following equation [9,11]:

$$G(\tau) = G(0) \sum_i \left(1 + \frac{8D_i \tau}{z_0^2} \right)^{-1} \left(1 + \frac{8D_i \tau}{z_0^2} \right)^{-1/2}$$  \hspace{1cm} (2)

Here $\tau$ is the lag time, $D$ is the diffusion constant of the quantum dot, $r_o$ is the laser beam radius at its focus and $z_0$ is the $1/e^2$ radius in the $z$ direction. The sum is over the distinguishable species, $i$, in solution. The TPE excitation volume $V = (\pi/2)^{1/2}r_o^2z_0$ was calibrated and found to be $30\,\text{fl}$ ($r_o = 1.4 \times 10^{-6}\,\text{m}$ and $z_0 = 5.3 \times 10^{-6}\,\text{m}$). The long working distance objective used in this study makes a larger excitation volume than effected with a more typical high numerical aperture lenses.

For a solution which has a distribution of NPs, $i$, the $G(0)$ can be described by the following equation,
where $\eta_i$ is the brightness of species $i$ in units of fluorescence count rate per particle.

Cross-correlation decays are modeled as above using [9,11]:

$$G(t) = G(0) \sum_i \left( 1 + \frac{8D_i \tau}{r_0^4} \right)^{-1} \left( 1 + \frac{8D_i \tau}{z_0^4} \right)^{-1/2}$$

The equations contain no terms to account for quantum dot blinking, the effects of which were minimized here by keeping the excitation rates low. When a low excitation rates does not remove the anomalous shape, we used the following equation, which helps account for blinking [8]:

$$G(t)^{\text{Blink}} = (G(t)) \left( 1 + \frac{F \cdot g_{\text{Blink}}(t)}{1 - F} \right)$$

Here $G(t)$ is the defined by Eq. (2). $F$ is the fraction of qdots with detectable fluctuations that cannot be described by the diffusion (allowed to float in fitting). $g_{\text{Blink}}(t)$ is a function that models the shape of the autocorrelation decay. Experimentally, $g_{\text{Blink}}(t)$ was found to be best defined as [8]:

$$g_{\text{Blink}}(t) = A(\tau)^{x-2}$$

where $A$ and $x$ are parameters allowed to float in fitting routines. It should be noted that this blink term does not influence the diffusion coefficient values that are obtained from the autocorrelation function fit. The term does allow for a more consistent concentration determination. Furthermore, using a low numerical aperture objective lens (0.4 NA) or very low powers, the blinking effect is not evident in ACDs.

### 2.3.2. Experimental set-up for fluorescence correlation spectroscopy

All qdots were obtained from Quantum Dot Corporation, QDC (Hayward, CA)/Invitrogen (Burlington, ON) with prefabricated surface coatings. For diffusion coefficient measurements, qdot samples were diluted to appropriate concentrations (1–20 nM) in ultrapure water (Millipore distilled) or phosphate buffered saline (PBS). Chicken blood serum (CBS) for both autofluorescence emission and diffusion coefficient experiments was obtained from US Biological (Swampscott, MA). Whole chicken blood for autofluorescence experiments was obtained from Charles River Laboratories (Wilmington, MA). Streptavidin-functionalized fluorospheres and carboxylate-terminated fluorospheres were obtained from Invitrogen.

#### 2.3.2.1. CAM preparation and micro-injection protocols

The chicken embryo chorioallantoic membrane was prepared according to Samkoe et al. [25]. With this in ovo procedure, one has access to blood vessels in a diameter range of 20–200 μm. The windowed eggs were mounted to a Zeiss Axioplan 200 microscope using an in-house stage to accommodate the eggs and allow micro-injection without need of refocusing the sample. Injections into flowing blood vessels were performed as previously described [25], with some minor modifications. In some cases, FCS measurements were facilitated by blocking blood flow with small air bubbles. To do this, air bubbles were back flushed into the micro-injection apparatus in small volumes (1–2 μL) between larger volumes of qdot solution (5–10 μL). When injected, these air bubbles arrested the flow of blood in small to medium sized vessels (with 20–100 μm inner diameter). When air bubbles were injected into veins, blood could be observed flowing in from smaller vessels but after the chick was cooled for ~15 min this blood flow also halted. An example of a venule with an air bubble to halt flow is shown in Fig. 1. Long straight blood vessels that follow the surface of the CAM were ideal for this purpose. It is difficult to control the positioning of air bubbles within the vessels so the use of vessels that remain superficial enables more choice for FCS measurements. Very small blood vessels (less than ~30 μm diameter) can be difficult to identify when filled with clear solutions since their narrow walls do not scatter much of the white light used for imaging. This can make focusing the laser excitation volume within these blood vessels difficult.

#### 2.3.2.2. Fluorescence correlation spectroscopy instrumentation

For TPE, we used a Spectra Physics (Palo Alto, CA) Titanium Sapphire Tsunami Laser operating at 780 nm and 82 MHz with 100 fs pulses. In ovo monitoring was performed using a 1-cm working distance, 20× Zeiss objective with 0.4 numerical aperture (NA) was used which has a 35 fL TPE volume for a back-aperture power of 100 mW [25]. Images and injections were performed under white light (using a StockerYale MilleLuce M1000 Gooseneck lamp with adjustable intensity). A 1.2NA, 100×, oil immersion objective (with excitation volume of approximately 1.25 fL for a back-aperture power of 30 mW) was used for diffusion coefficient determination of qdots in various biological media. A neutral density filter wheel was used to adjust the laser intensity reaching the sample. Autocorrelation decays were generated using an ALV500/E correlator card (Langen, Germany) and fitted using ORIGINPRO 7.0 software.

#### 2.3.3. NPs and blood sera

An investigation of the interaction of NPs with blood serum and with whole blood is essential to understand NPs in vivo, because the diffusion of NPs may be influenced by their interaction with blood proteins and blood cells. In this study, we aimed to examine NP diffusion within blood sera. In addition, the diffusion coefficients determined by FCS provided an estimate of the NP hydrodynamic size and therefore could be used to determine whether binding occurred between NPs and proteins and indicate states of NP–NP aggregation. Protein–NP interactions have the capacity to influence NP uptake and localization in the chorioallantoic membrane (CAM). Moreover, the diffusion coefficients determined in blood serum could approximate the diffusion of NPs within blood vessels of the CAM. These estimates can be used to help fit to NP data that may include more phenomena such as flow and
or concentration changes. In general, it is necessary to investigate behavior in a biologically simplified system so that deviations from the behavior in the simplified system may provide insight into NP interactions with tissue in ovo.

Much of the data was gathered for qdots as test NPs. Functionalized CdSe/ZnS core/shell qdots are readily available in high quality from commercial suppliers. Qdots with emission maxima at 525, 565, 605 and 655 nm were used for the experiments described here. To understand the diffusion characteristics of qdots, it is important to estimate their size. Spherical CdSe NPs with photoluminescence spectra maxima ranging from 470 to 625 nm have an estimated core diameter of 2.3–5.5 nm [29]. Each ZnS monolayer added to a CdSe core is 3.1 Å thick [29], and qdots typically have 1–2 monolayers of the capping material. This shell increases the radius of the 470–625 nm emitting qdots to the range of 1.3–3.1 nm. The greatest contribution to qdot size is the polymer coating required to make NPs water soluble. Invitrogen-produced water soluble amino-, streptavidin-, and methoxy-conjugated qdots are coated with PEG polymers to which functional groups/molecules are attached [30]. Carboxylated qdots are known to have a polyacrylic acid (PAA) surface coating. Streptavidin qdots are made with PEG–2000 and the length of polymers on other qdots is not available from the manufacturer, but the polymer greatly increases the qdot diameter. Pons et al. [31] characterized commercially available qdots by dynamic light scattering and found the calculated radius of a 565 nm emitting carboxylate qdot obtained from Invitrogen to be 7.1 ± 0.2 nm. Ryman-Rasmussen et al. [18] note the hydrodynamic radii of carboxy855 qdots and methoxy565 qdots as being 18 and 35 nm, respectively. Invitrogen does not provide information about the radii of the qdots in each production batch. Thus, it is essential to characterize the radii of purchased qdots before they can be used in ovo.

For FCS measurements, qdots are assumed to be spherical in shape although imperfections in qdot synthesis may lead to non-spherical qdots. Furthermore, it is known that 655 nm emitting qdots from qdot/C/Invitrogen are more ovoid in shape than spherical [30] and have major axes that are approximately twice that of their minor axes. Li et al. [32] provide an investigation into shape manipulation of CdSe quantum rods showing that the band gap is highly dependent on qdot shape. Since a spherical qdot’s band gap can be closely matched to that of a rod-shaped NP by changing the aspect ratio, the emission maxima alone cannot confirm their core dimensions.

Further complications for the spherical qdot model exist by adding proteins, such as streptavidin, to qdot surfaces. In its biologically active conformation, streptavidin has dimensions of 5.4 nm × 5.8 nm × 4.8 nm [33]. The binding of one monolayer of streptavidin onto a visible-emitting, polymer-coated qdot could potentially double the largest qdot dimension, thus, distorting the overall spherical shape of the molecule. The possibility of multiple streptavidin molecules conjugated to a single qdot also discards the idea of a spherical qdot. In fact, literature suggests that more than one streptavidin molecule is attached to each streptavidin functionalized qdot [9] obtained from Invitrogen. Nevertheless, estimates of qdot diffusion coefficients that were determined using FCS were used to estimate hydrodynamic radii here and in literature [9,13,16,34]. These estimates could be applied to fitting procedures for future, more complex biological systems to enable estimates of concentration and flow. FCS has also been used to estimate qdot concentrations in literature [13] and qdots have previously been used to estimate blood flow in mature rats in vivo [12].

2.3.3.1. Quantum dot diffusion in various buffers. The diffusion characteristics of qdots in various media were compared to determine whether or not proteins in animal blood serum interact with qdots of various surface functionalizations. The diffusion coefficients determined by FCS provide an estimate of the qdot hydrodynamic sizes (including surface ligands, polymer coatings and ZnS shell) and could be expected to approximate the diffusion of qdots within a blood vessel of the CAM if no further qdot interactions take place.

The viscosities of water and chicken blood serum (CBS) were used to calculate the hydrodynamic radii of qdots. The viscosity of water at room temperature was taken to be 1.002 cP and the viscosity of CBS was determined to be 1.26 ± 0.02 cP at room temperature (22 °C) using a calibrated viscometer. This value was very similar to the viscosities of human sera [35] and broiler chicken plasma [36] listed in literature. Although viscosity changes with temperature [37], the laboratory temperature was thought to be stable over time, so radii could be calculated from FCS data using this viscosity.

Autocorrelation decays (ACDs) were taken in phosphate buffered saline (PBS), CBS, rabbit blood serum (RBS) and water; they were fitted using Eq. (5), which accounts for the anomalous shape of the ACD due to blinking. Runs with unstable count rate trajectories were discarded at the point of collection since these ACDs often correspond to the presence of large qdot aggregates in solution. A number of diffusion coefficients were averaged to determine the diffusion coefficient in each solvent. An example of a typical fitted ACD and corresponding count rate trajectory are shown in Fig. 2.

It should be noted that when the additional ‘blink’ term (Eq. (5)) was used to fit autocorrelation decays with the characteristic qdot short-time anomalous shape, the value of the fitted diffusion coefficient was not significantly influenced [9]. This was verified by a comparison of diffusion coefficients fitting with the blink term and fitting without the blink term.

The diffusion coefficients of qdots in chicken and rabbit blood sera tended to be smaller than those in PBS and water. A summary of the measured diffusion coefficients is provided in Fig. 3. The higher viscosities of the blood sera were expected to account for these differences. The Stokes–Einstein equation was used to calculate the radii of qdots:

\[
D = \frac{kT}{6\pi\eta R}
\]

where \(D\) is the diffusion coefficient (m²/s) determined by FCS, \(T\) is the temperature (K), \(k\) is the Boltzmann constant (1.38065 × 10⁻²³ m² kg s⁻² K⁻¹), \(\eta\) is the viscosity of the solvent (P) and \(R\) is the radius (m).

It is clear that the qdot coating shell has a large influence on the NP diameter. Smaller qdots would be expected to have a larger diffusion coefficient. The more red the qdot emission, the larger the nanocrystal core. However, from examination of the measured hydrodynamic radii in Fig. 4, there is little relationship observed between overall radius and size of core, suggesting that the polymer coatings largely determine the qdot size. The measured sizes of Invitrogen qdots are similar to those measured using dynamic light scattering [31] and FCS (for streptavidin functionalized qdots) [9].

It can be seen that the measured radii in water and CBS are within error for all the qdots measured, except for those with carboxylated coatings. A Students T test (Table 1) confirms that the radii are not significantly different in CBS and water with 95% confidence, except for carboxylate coated qdots.

This greater measured radius in CBS may be evidence of interaction between proteins in CBS and carboxyylate coated qdots or simply of qdot–qdot aggregation. We have performed FCSs experiments on solutions containing qdots and fluorescence labelled BSA and found no evidence of association. With respect to aggregation, groups of two or three attached qdots that move together through
the excitation volume would decrease the measured diffusion coefficient. Further evidence to suggest that this is not the case is shown in Fig. 5. The same dilution of carboxy605 stock solution was made in CBS and water by serial dilutions of large volumes (50 or more L). It can be seen that the \( G(0) \) value and fitted concentration is very similar for both aliquots, however, the decay in the ACD is shifted to a longer time scale for the qdots diluted in CBS. The diffusion coefficient in water was determined at two and a half times that measured in CBS for the ACDs in Fig. 5. In the case of aggregation, one would expect a higher \( G(0) \) owing to a dilution of the total number of diffusing particles. These similar \( G(0) \) values are evidence to suggest that the increase diffusion coefficient is a hydrodynamic difference between the solutions and not the result of aggregation. It may be that particular polymer coatings of qdots change size when in solutions of high ionic strength.

The difference in measured hydrodynamic radii between carboxylated qdots in water and CBS is hypothesized to be related to the increased ionic strength of CBS. In order to investigate the response of carboxylated qdots to solutions of increasing ionic strength, carboxy655 qdots were diluted in solutions of 300–100 mM PBS. Autocorrelation decays were acquired and used to determine the diffusion coefficients of qdots as a function of ionic strength. The viscosity of each PBS solution was measured and used to calculate the hydrodynamic radii, using the Stokes–Einstein equation (Eq. (7)). The averages of eight calculated radii per PBS solution are shown in Fig. 6. These calculated radii are compared to the radii measured in pure water and in CBS.

Since the calculated hydrodynamic radii account for viscosity differences between the solutions, it is clear that the concentration of ions within solution has an addition effect on the hydrodynamic radii of carboxy-qdots. Carboxylated qdots from Invitrogen/QDC are noted in literature as being composed of polyacrylic acid (PAA) [30], which is known to swell under high ionic strength conditions [38]. Chibowski et al. noted an increase in the thickness of a

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**Fig. 2.** (A) An example of an autocorrelation decay used to determine the diffusion coefficient of amino605 QDs in water. The diffusion coefficient was determined to be \((7.80 \pm 0.14) \times 10^{-13} \text{ m}^2/\text{s}\) and the concentration was determined to be \((2.35 \pm 0.02) \times 10^{-9} \text{ M}\). The red line is a fit using the blinking equation (Eq. (5)). (B) The corresponding count rate trajectory for the same ACD.

**Fig. 3.** Diffusion coefficients of various QDs in four different solvents determined by fluorescence correlation spectroscopy. Water, phosphate buffered saline, chicken blood serum and rabbit blood serum were used for all experiments. Amino (A), biotin (B), carboxy (C), methoxy (M) and streptavidin (S) functionalized QDs were used for the measurements. The number on the x-axis denotes the emission wavelength of the QD.
PAA adsorption layer on aluminum oxide [38]. This increased thickness was thought to result from a screening effect charges on the polymer segment by ions from the solution. The decreased repulsive forces between the polymer chain segments causes the PAA to become coiled on the surface. This leads to a conformation of polymer rich in loops and thus increases the hydrodynamic radius of encapsulated particles. In addition, at high electrolyte concentrations can cause sodium ions to occupy active sites on the surface of aluminum oxide. This forces the surrounding polymer to stretch out into the solution, increasing the size of the particle. Perhaps a similar effect occurs at the ZnS/PAA interface of qdots. Carboxylate qdots may also be sensitive to the pH of solution. As pH decreases the proportion of protonated carboxylate increases and this causes the PAA chains to repel in solution, thus, their conformation is expanded in alkali media. Therefore, pH changes may significantly change the diffusion of carboxylated qdots.

The count rate per particle was calculated from the average of count rate fluorescence trajectories and the concentration from the fitted ACDs used to determine the diffusion coefficients. These results are summarized in Fig. 7. Since the count rate per qdot is relatively stable across different media, this suggests that no significant quenching effects are occurring in chicken blood serum. In addition to confirming the qdot surface stability in blood sera, this also partially validates the use of count rate fluorescence trajectories as an estimate of qdot concentration in solution, since fluorescence intensity is linearly proportional to concentration.

In future work it would be advantageous to create a more physiologically relevant ionic solution with the most common ions in concentrations similar to those in CBS. Then the deviations in measured hydrodynamic radii in vivo could be attributed to qdot–protein interactions and/or aggregation.

### Table 1
Students T test calculations on calculated radii of various qdots.

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Calculated radius in water (nm)</th>
<th>Calculated radius in CBS (nm)</th>
<th>(n) water</th>
<th>(n) CBS</th>
<th>(T_{stat}) value</th>
<th>(T_{critical}) value(^b)</th>
<th>(T_{critical}) value(^b) (95%)</th>
<th>Radius in CBS significantly greater than radius in water?</th>
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</thead>
<tbody>
<tr>
<td>QD Amino525</td>
<td>8.2 ± 1.5</td>
<td>9.7 ± 2.0</td>
<td>8</td>
<td>8</td>
<td>1.71</td>
<td>1.75</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>QD Amino605</td>
<td>5.7 ± 2.1</td>
<td>7.4 ± 3.9</td>
<td>8</td>
<td>8</td>
<td>1.09</td>
<td>1.76</td>
<td>No</td>
<td></td>
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<tr>
<td>QD Amino655</td>
<td>7.9 ± 2.7</td>
<td>8.9 ± 2.1</td>
<td>8</td>
<td>8</td>
<td>0.89</td>
<td>1.73</td>
<td>No</td>
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<tr>
<td>QD Biotin605</td>
<td>21.9 ± 2.4</td>
<td>20.3 ± 3.9</td>
<td>4</td>
<td>6</td>
<td>-0.71</td>
<td>2.31</td>
<td>No</td>
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<tr>
<td>QD Carboxyl525</td>
<td>3.8 ± 0.7</td>
<td>9.4 ± 1.7</td>
<td>8</td>
<td>8</td>
<td>8.51</td>
<td>1.76</td>
<td>Yes</td>
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<tr>
<td>QD Carboxyl605</td>
<td>7.1 ± 1.4</td>
<td>13.8 ± 3.2</td>
<td>8</td>
<td>8</td>
<td>5.40</td>
<td>1.76</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>QD Carboxyl655</td>
<td>5.5 ± 1.5</td>
<td>12.7 ± 4.4</td>
<td>10</td>
<td>10</td>
<td>4.85</td>
<td>1.73</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>QD Methoxy565</td>
<td>10.3 ± 2.3</td>
<td>12.0 ± 2.9</td>
<td>8</td>
<td>8</td>
<td>1.29</td>
<td>1.76</td>
<td>No</td>
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<tr>
<td>QD Strep605</td>
<td>13.2 ± 2.7</td>
<td>12.4 ± 4.2</td>
<td>7</td>
<td>8</td>
<td>-0.42</td>
<td>1.77</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Fluosphere-20</td>
<td>8 ± 1</td>
<td>22 ± 2</td>
<td>8</td>
<td>8</td>
<td>9.3</td>
<td>1.8</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) Note that \(n\) denotes the number of values averaged.

\(b\) Note that if \(T_{stat} < T_{critical}\) then the hydrodynamic radius measured in CBS is not greater than the hydrodynamic radius measured in water.

**Fig. 4.** Calculated radii of various QDs in chicken blood serum and in water. The vertical bars represent the error. Radii were calculated using the Stokes–Einstein equation and the fitted diffusion coefficient determined by fluorescence correlation spectroscopy. The radii of carboxylated QDs are significantly different in water and CBS. The radii of all other QDs are not significantly different in CBS compared to water.

**2.3.3.2. Quantum dot diffusion in whole chicken blood.** In non-flowing whole chicken blood, FCS measurements of qdots are difficult to collect. The inhomogeneity of blood cells and qdots in the blood serum as the blood cells settle to the base of the well-slide makes the fluorescence trajectories unstable and unpredictable. Over collection periods as short as 3 s, the fluctuations in fluorescence intensity are still too inconsistent to collect an acceptable autocorrelation decay. Over long time scales there are large aggregates of qdots that appear to pass through the excitation volume. After 20 min of autocorrelation analysis, the average fluorescence intensity drops to approximately 4.5 kHz, comparable with the autofluorescence signal of chicken blood serum. Over time, red blood cells and particulate matter in chicken blood was seen to descend to the bottom of a solution. Moreover, this settling effect appears to remove the qdots from their suspension in solution, evident by the decrease in count rate over time.
2.3.4. Quantum dot solution in flowing blood vessels: bolus distribution

In the CAM vessels, the increased turbulence of flowing blood allows a more uniform distribution of qdots amongst the blood cells. This would maintain a steadier fluorescence trajectory and make the collection of autocorrelation decays within a blood vessel possible. The redistribution of the qdot bolus within the chick embryo blood vessel system appeared to occur on a short time scale since after injection of 5–20 \( \mu \)L of 30 nM carboxy605 qdot solution, a second bolus of fluorescent material was not observed, suggesting that the bolus had dissipated (Fig. 8). This could be related to the low concentrations and volumes used. For larger volume and higher concentration injections, it may be possible to observe a diminished bolus in recirculation and thus estimate the circulation time.

The circulation time of blood within the chicken embryo was of interest, since it may be possible to measure qdot dynamics as the qdot injection bolus passes through a blood vessel area. This would benefit experimentalists as it would provide an analytic technique to monitor qdot dynamics while using a smaller volume of the commercially expensive qdots. In order to determine the approximate circulation time of a bolus within the chicken embryo circulation, FluoSpheres were used, since they can be used in high quantities at a relatively low cost. An injection of 20 \( \mu \)L of a concentrated (1.9 \( \times \) 10\(^{-8}\) M concentration) 100 nm diameter FluoSphere solution into a vein of the CAM and subsequent monitoring of fluorescence intensity reveals that the remnants of the injection bolus return to the same blood vessel after approximately 20 s. This can be seen in Fig. 9 and was observed for a range of qdots with diameters <500 nm.

The fluorescence trajectory of the initial bolus downstream of the injection site after one cycle of blood circulation is irregular, which makes the collection of autocorrelation functions challenging. Some examples of 3-s-long ACDs of carboxy605 qdots taken during the first and second circulation of a large bolus of qdot solution (following a 60 \( \mu \)L of a 10 nM carboxy605 qdot solution) are shown in Fig. 10. It is clear that the ACDs are irregular and this is attributed to the widely fluctuating count rate trajectories resulting from the inhomogeneity of the qdot bolus immediately after injection and following partial dissipation within the circulating blood. Consequently, systemic (i.e. venous) injections were used since they provided a more even distribution of spheres and qdot within the blood vessel system.

2.3.5. Quantum dot diffusion in vessels of the chorioallantoic membrane: stopped flow

One final consideration when using the CAM as a blood vessel model for FCS applications, is the diffraction and reflection of the
two-photon excitation and fluorescence emission within the CAM tissue. The longer wavelength (780 nm) of light used in TPE decreases the scattering light, however, the emitted fluorescence at longer wavelengths may be more largely influenced by scattering effects. The absorption spectra of CBS, CAM and whole chicken blood all show two small absorbance peaks around 525 nm. Therefore, from a practicality point of view, the use of qdots of longer emission wavelength may lead to less scattering and absorption of emitted fluorescence. In deep vessels, however; the effect of scattering and absorption on both autocorrelation functions and on fluorescence intensity should be considered, especially if autocorrelation functions are to be used for qdot concentration comparisons across different blood vessels and qdot types.

A ‘stopped flow’ injection (described in Section 2) can be used to inject a small volume of qdot solution into a CAM blood vessel between two air bubbles. This solution can then be compared with the same solution that is prepared in a slide. A series of experiments were carried out in this way using carboxy605, biotin605, amino525, strep605 and strep525 qdots. An example of the results of averaged autocorrelation functions and count rate trajectories is shown in Fig. 11 and summarized in Table 2.

By comparing the autocorrelation decays of qdot solutions in blood vessels and in a sealed microscope well-slide, it appears that the diffusion coefficient behavior of qdot solutions is not greatly changed in ovo. In some cases, the concentration of the qdot solution appears to be lower when in ovo than when in a well-slide. The change in concentration may result from aggregation of qdots in the injection apparatus prior to injection or absorption of some of the qdot particles to the blood vessel walls. The observed fluorescence count rate of a qdot solution in ovo is often lower than that of the same solution within a well-slide under the same experimental parameters. The scattering of excitation and of fluorescence by biological tissue is expected to account for the lower in ovo count rate per qdot observed in the trajectories.
A summary of compared concentrations, average fluorescence intensity and diffusion coefficients are shown in Table 2. From these results it is clear that the well-slide and CAM measurements are very similar. That is with the exception of the 525 nm emitting qdots, which have significantly attenuated fluorescence when in the CAM. This can be rationalized by reabsorption of the 525 nm fluorescence by the CAM tissues, because they display a significant absorbance feature in that region of the spectrum.

### Table 2

<table>
<thead>
<tr>
<th>Quantum dot</th>
<th>Amino525</th>
<th>Biotin605</th>
<th>Carboxy605</th>
<th>Strept525</th>
<th>Strept605</th>
</tr>
</thead>
<tbody>
<tr>
<td>Approximate Venule inner diameter (μm)</td>
<td>120</td>
<td>60</td>
<td>60</td>
<td>80</td>
<td>50</td>
</tr>
<tr>
<td>Diffusion coefficient in vessel (×10⁻¹² m²/s)</td>
<td>4.8 ± 0.5</td>
<td>3.4 ± 0.2</td>
<td>7.6 ± 1.2</td>
<td>3.6 ± 1.0</td>
<td>4.4 ± 0.9</td>
</tr>
<tr>
<td>Diffusion coefficient in slide (×10⁻¹² m²/s)</td>
<td>4.0 ± 0.1</td>
<td>2.5 ± 0.4</td>
<td>4.7 ± 0.7</td>
<td>5.1 ± 0.2</td>
<td>5.8 ± 0.6</td>
</tr>
<tr>
<td>Concentration in vessel (×10⁻¹⁰ M)</td>
<td>4.9 ± 0.1</td>
<td>5.1 ± 0.1</td>
<td>3.9 ± 0.1</td>
<td>7.8 ± 0.5</td>
<td>7.5 ± 0.3</td>
</tr>
<tr>
<td>Concentration in slide (×10⁻¹⁰ M)</td>
<td>6.1 ± 0.4</td>
<td>5.8 ± 0.2</td>
<td>4.5 ± 0.1</td>
<td>8.6 ± 0.2</td>
<td>9.9 ± 0.2</td>
</tr>
<tr>
<td>Average count rate in vessel (kHz)</td>
<td>1.3 ± 0.2</td>
<td>21.3 ± 1.6</td>
<td>16.0 ± 0.9</td>
<td>1.3 ± 0.2</td>
<td>20.5 ± 1.9</td>
</tr>
<tr>
<td>Average count rate in slide (kHz)</td>
<td>3.0 ± 0.3</td>
<td>20.2 ± 0.6</td>
<td>16.3 ± 0.7</td>
<td>6.8 ± 0.9</td>
<td>34.2 ± 2.0</td>
</tr>
</tbody>
</table>

2.3.6. Nanoparticle diffusion in vessels of the chorioallantoic membrane: flowing blood

To measure NPs in the blood stream of a living embryo, systemic injections of qdots into large vessels (100–150 μm diameter) were performed and autocorrelation decays were measured significantly downstream from the injection site and in smaller vessels (50–120 μm). A 5-min post-injection dispersion time was found to be optimal for FCS measurements. A time series of
autocorrelation decays (ACDs) was used to measure the qdot blood concentration versus time. A measured change in the fluorescence count rate in blood could also be used as an indirect estimate of decreasing qdot concentration, but this would not report on aggregation state or changes in fluorescence yield. The consequences of quantum dots leaving the blood stream will be reported elsewhere.

Fig. 12. Data resulting from fluorescence of a 200 mL injection of $5 \times 10^{-3}$ M polystyrene nanosphere (40 nm diameter) into the blood vessel system of the CAM, without evidence of aggregation, panels (a), (b) and (c); and with aggregation (d), (e) and (f). (a) Autocorrelation decay. (b) Count rate trajectory. (c) Count rate distribution. (d) Autocorrelation decay. (e) Count rate trajectory, showing intensity spikes of aggregates. (f) Count rate distribution, again displaying evidence of larger intensities from aggregated nanospheres.
Returning to aggregation, FCS can report on aggregation of NP solutions in vivo or ex vivo. With FCS it is possible to report on the degree of aggregation, through analysis of the autocorrelation decays (Eqs. (2) and (3)). For example, the panels in Fig. 12 display comparisons of polymer nanosphere (40 nm diameter) solutions injected into the CAM. In one case, Fig. 12a–c, no aggregation was observed. In another case, Fig. 12d–f, aggregation was evident. The two injections were of precisely the same nanosphere solution and thus one knows the original contents of the solution.

A lack of aggregation is verified from the value of $G(0)$ in Fig. 12a. Since $1/G(0)$ is the number of emitters in the TPE volume, this value can be predicted knowing the injection concentration and the approximate blood volume. Two-hundred microliters of a $5 \times 10^{-5}$ M solution was injected into a large blood vessel (150 μm diameter). The apparent concentrations in the inyectates will decrease by a factor of approximately 2, through dilution into the blood volume. From the $G(0)$ of 0.029 (particle/volume)$^{-1}$ and a calibrated TPE volume of 35 fL, we calculate the concentration to be $1.7 \times 10^{-8}$ M. This compares well with the predicted concentration of $2.5 \times 10^{-8}$ M. By contrast, Fig. 12d shows a $G(0)$ of 0.18 (particle/volume)$^{-1}$ which delivers a concentration of $0.24 \times 10^{-5}$ M, and suggests loss through aggregation. This is supported by changes in the peaks in count rate trajectory plots (Fig. 12b and e), where the peak count rates double for the aggregated sample. The changes in count rate trajectories suggest that in the aggregated sample there is likely a combination of free particles and aggregates.

One of the advantages of FCS is that the data can be analyzed to help determine the degree of aggregation and particle distributions in solution. For the non-aggregated data set in Fig. 12a–c one can determine the average brightness (fluorescence count rate per NP) of the NPs by multiplying average count rate from Fig. 12c, by $G(0)$ from Fig. 12a. This brightness can then be used as the quantum of fluorescence produced by each nanosphere in the aggregate (presuming the brightness does not change upon aggregation). Also, to a first approximation mass balance will exist between pre- and post-aggregation, which can be used as a constraint when fitting Eqs. (2) and (3) to the data produced by an aggregated sample. In the case of the nanospheres, aggregation appears increase over time and is more severe in smaller blood vessels.

For the nanosphere sample in the above figures, the average brightness can be calculated: $4.1 \text{ kHz} \times 0.029 \text{ particle}^{-1} = 0.12 \text{ kHz/particle}$, which is $\eta_1$ in Eq. (3). In the experiment where aggregation was observed, the $G(0)$ increased to 0.18 particle$^{-1}$, indicating fewer total particles and the count rate had spikes in the range 6–12 kHz. We chose $1.8 \text{ kHz/aggregate}$ as starting point indicating fewer total particles and the count rate had spikes in concentration state of fluorescent NPs. We have found that in smaller blood vessels (diameter <60 μm) more aggregation takes place than in larger blood vessels (100–150 μm diameter), particularly for NPs with QDs on their surfaces. These findings are important because aggregation state likely influences NP accumulation in angiogenic tissues. Using FCS, it will be possible to measure the loss of NPs from the blood streams of live embryos. The kinetics of loss could be correlated with surface charge, chemistry and size and the rate constants inputted into predictive models. These models would be essential for governmental regulators to make sound decisions on nanomaterials.

### Acknowledgements

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### References

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