New Tools for Real-Time Study of Embryonic Development

Lauren M. Browning

Old Dominion University

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NEW TOOLS FOR REAL-TIME STUDY OF EMBRYONIC DEVELOPMENT

by

Lauren M. Browning
B.S. May 2006, Old Dominion University – Norfolk, VA

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Old Dominion University in Partial Fulfillment of the
Requirements for the Degree of

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BIOMEDICAL SCIENCES

OLD DOMINION UNIVERSITY
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ABSTRACT

NEW TOOLS FOR REAL-TIME STUDY OF EMBRYONIC DEVELOPMENT

Lauren M. Browning
Old Dominion University, 2013
Director: Dr. Xiao-Hong Nancy Xu

Embryonic development represents one of the most complex and dynamic cellular processes in biology, and plays vital roles in understanding of functions of embryonic stem cells (ESCs) and design of ESC-based therapy. Conventional assays and fluorescence-based imaging methods have been widely used for the study of embryonic development. These conventional methods cannot effectively provide spatial and temporal resolutions with sufficient sensitivity and selectivity that are required to depict embryonic development in vivo in real-time at single-cell and single-molecule resolutions. In this dissertation, we have developed a wide range of innovative tools for real-time study of embryonic development. These new tools include biocompatible and photostable plasmonic gold (Au) and silver (Ag) nanoparticle (NP) imaging probes, dark-field optical microscopy and spectroscopy (DFOMS), and ultrashort electric pulses. We have designed and synthesized a mini-library of Au and Ag NPs with different sizes and chemical properties. We have used developing zebrafish embryos as in vivo model organisms to study embryonic development and as in vivo assays to study size- and chemical-dependent nanotoxicity. We found that these multicolored imaging probes can passively diffuse into embryos and enter into embryos non-invasively. These NPs exhibit superior photostability and enable us to study embryonic environments for a desired period of time. They can be illuminated under a standard microscope halogen lamp and characterized simultaneously using DFOMS equipped with a multi-spectral imaging system to achieve real-time multiplexing imaging. Our studies show that Au NPs are much more biocompatible than Ag NPs, while Ag NPs are much more sensitive and colorful than Au NPs. Notably, we can make Ag NPs nearly as biocompatible as Au NPs by functionalizing their surfaces with biocompatible peptides. Furthermore, Ag
NPs can incite stage-specific embryonic phenotypes, and enable us to generate distinctive mutants for further identification of biomarkers for better understanding of embryonic development and for potential diagnosis of birth defects. We have developed new methods to effectively culture and sustain ESCs of zebrafish, mouse and human, laying down the foundation for real-time study of differentiation of ESCs both in vitro and in vivo for a wide variety of biomedical applications.
This dissertation is dedicated to my parents, Mr. and Mrs. Steve and Linda Browning for their unrelenting love and support, which made all of this possible and to my daughter, Caroline Marie Boatright, who has made me a stronger woman.
ACKNOWLEDGMENTS

First and foremost, I would like to thank my mentor, Dr. X. Nancy Xu for her dedicated assistance and support throughout my Ph.D. study at Old Dominion University. She has given me the opportunity to explore, design, create and achieve unique scientific research. This dissertation would not be possible if it wasn't for her devotion to me and her faith in me and my ability to conduct cutting-edge research. I am grateful for her for giving me the chance to learn new and different scientific techniques and multiple disciplines of the sciences. She has inspired me to become more confident as a researcher.

I would like to extend thanks to the members of my dissertation committee, Drs. Kenneth Brown, Lesley H. Greene, Christopher J. Osgood, and Bala Ramjee for their helpful knowledge and support through my graduate work. In addition, I would like to acknowledge my fellow lab group members, especially Kerry J. Lee, Prakash D. Nallathamby and Dr. Tao Huang, for their dedication and contribution to this dissertation.

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NOMENCLATURE

ε  Extinction coefficient
λ_{max}  Peak wavelength
µm  Micrometer
µM  Micromolar
µg  Microgram
µL  Microliter
µs  Microsecond(s)
η  Viscosity
γ  Gamma
a  Radii of single nanoparticles
ALP  Alkaline phosphatase
Ag  Silver
AgNO_{3}  Silver nitrate
Au  Gold
BMP  Bone morphogenetic protein
BSA  Bovine serum albumin
cm  Centimeter
C  Concentration
CCD  Charge coupled device
CLs  Chorion Layers
CS  Chorion space
CO_{2}  Carbon dioxide
<table>
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<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>D</td>
<td>Diffusion coefficient</td>
</tr>
<tr>
<td>d</td>
<td>Density</td>
</tr>
<tr>
<td>DFOMS</td>
<td>Dark field optical microscopy and spectroscopy</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagles medium</td>
</tr>
<tr>
<td>E</td>
<td>Electric field strength</td>
</tr>
<tr>
<td>EBs</td>
<td>Embryoid bodies</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMCCD</td>
<td>Electron multiplying charge coupled device</td>
</tr>
<tr>
<td>ESCs</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>ES-DMEM</td>
<td>ESC qualified Dulbecco’s modified Eagles medium</td>
</tr>
<tr>
<td>ES-FBS</td>
<td>ESC qualified Fetal Bovine Serum</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FL</td>
<td>Feeder layer</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width at half maximum</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Water</td>
</tr>
<tr>
<td>HAuCl&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Hydrogen tetrachloroaurate (III)</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours-post-fertilization</td>
</tr>
<tr>
<td>HR-TEM</td>
<td>High-resolution transmission electron microscopy</td>
</tr>
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</table>
IME  Inner mass of embryo
IRR  Irradiated
k    Boltzmann constant
kV   Kilovolts
L    Liter
LIF  Leukemia inhibitory factor
LSPR Localized surface plasmon resonance
M    Molarity
MAB  Monoclonal antibody
MEFs Mouse embryonic fibroblasts
mESCs Mouse embryonic stem cells
MΩ   Megaohms
min  Minute
mL   Milliliter
mm   Millimeter
mM   Millimolar
mol  Mole
ms   Millisecond
MSD  mean-square-displacement
NaCl Sodium chloride
nM   Nanomolar
nm   Nanometer
NPs  Nanoparticles
ns   Nanoseconds
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<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>OC</td>
<td>Outside chorion</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PHOTON</td>
<td>Photostable optical nanoscopy</td>
</tr>
<tr>
<td>PEFs</td>
<td>Pulsed electric fields</td>
</tr>
<tr>
<td>pM</td>
<td>Picomolar</td>
</tr>
<tr>
<td>QDs</td>
<td>Quantum dots</td>
</tr>
<tr>
<td>QY</td>
<td>Quantum yield</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RTSD</td>
<td>Real-time square-displacement</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SSEA-1</td>
<td>Stage-specific embryonic antigen-1</td>
</tr>
<tr>
<td>SSEA-4</td>
<td>Stage-specific embryonic antigen-4</td>
</tr>
<tr>
<td>T</td>
<td>Temperature in kelvin</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>UV-vis</td>
<td>Ultraviolet - visible</td>
</tr>
<tr>
<td>W</td>
<td>Watt</td>
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<tr>
<td>YS</td>
<td>Yolk sac</td>
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CHAPTER I

OVERVIEW

Conventional methods for in vitro and in vivo bioanalysis, including immunoassays, PCR and microarray based transcriptional analysis, have provided important ensemble information; however they have considerable drawbacks. These techniques do not allow for real-time analysis, hence critical knowledge about dynamic events of interest in living systems is lost. Furthermore, these methods do not offer the temporal and spatial resolutions required to elucidate the real-time molecular mechanisms and interactions occurring within living cellular environments. Additionally, most of these techniques are performed in bulk ensemble measurements. However, individual molecules, individual cells, and embryos can behave very differently. Thus, bulk measurements could miss crucial rare events of interest, which underscores the importance of developing new ultrasensitive molecular-based and real-time methods for study of single live cells. In this dissertation, we have developed a wide range of innovative tools, ranging from single NP optics, single NP imaging probes and in vivo nanotoxicology assays to ultrashort electric pulses and used them to study functions of single living cells and embryos in real-time. We have demonstrated that these new tools can potentially be used to address a wide variety of fundamental questions in biology and explore their applications in medicine, including how embryos develop, how to achieve and design target-specific drug delivery, and rationally design embryonic stem cells (ESCs) based therapies.

Over the past several years, a tremendous amount of attention and research has been devoted to nanomaterials and their potential applications in biology and medicine.¹ Nanomaterials are being explored as possible carriers for effective drug delivery and target therapy, and for biomedical imaging.¹⁻⁴ Among nanomaterials, noble metal NPs, such as Au and Ag NPs are the most interesting because their distinctive plasmonic and optical surface properties.¹⁻⁵

The journal for this dissertation is ACS Nano
Noble metal NPs have unique characteristics, such as size- and shape-dependent optical properties, a high surface area to volume ratio and surfaces that can be easily modified and functionalized with a variety of molecules. Additionally, their small size allows them to penetrate into living cells and organisms with ease. Thus, allowing the possibility of using them as photostable multicolored optical and photonic molecular probes and sensors for real-time imaging in living organisms.

The optical properties of noble metal NPs are attributed to a phenomenon called localized surface plasmon resonance (LSPR), which is highly dependent upon their size, shape and surrounding environments. Additionally, Au and Ag NPs exhibit strong Rayleigh scattering, allowing them to be directly observed in real-time and characterized using dark-field optical microscopy and spectroscopy (DFOMS) equipped with a multi-spectral imaging system (MSIS) to achieve multiplexing imaging. Since the multicolored NPs can be illuminated under a standard microscope halogen lamp there is no need for multiple single-wavelength excitation sources (e.g., lasers), which avoids auto-fluorescence of living organisms and significantly improves the signal-to-noise ratio. These exceptional properties have enabled noble metal NPs to become promising tools for various biomedical applications, including ESC differentiation.

During embryonic development, ESCs arise from the inner cell mass of the embryo that differentiate and develop into mature cells that have specific functions. These specialized cells offer possibilities of ESCs to be utilized as an endless supply of cells for drug screening and other cell-based therapies (e.g., cellular transplantation) to treat an array of diseases and disorders. Unfortunately, the molecular mechanisms underlying differentiation during embryonic development remain essentially unknown, due to the fact that current cellular assays and probes cannot effectively provide spatial and temporal resolutions with sufficient sensitivity and selectivity. Thus, the distinctive optical
and surface properties of NPs offer the possibility using them as an ultrasensitive tool to advance ESC research.

Nonetheless, before NPs can be used to study ESC differentiation during embryonic development \textit{in vivo}, several items need to be addressed. Firstly, the toxicological responses and an understanding of the mechanisms that control the behavior and fate of NPs upon introduction into a biological system are essential and necessary. Secondly, in order to lay the foundation for real-time \textit{in vivo} study of ESC differentiation, \textit{in vitro} studies need to be performed first. However, ESC cultures are extremely difficult and have challenges that need to be overcome.

In this dissertation, as a first step, we developed new imaging approaches using DFOMS-MSIS to study the transport of Au and Ag NPs into/in zebrafish embryos and assess their possible size- and chemical-dependent toxic effects. This research would offer new insights into the rational design of: (i) more biocompatible plasmonic NP imaging probes and (ii) therapies for early embryonic development. Thus, Au and Ag NPs can be potentially utilized as single NP imaging tools to study important processes occurring during embryonic development, including ESC differentiation, in living embryos in real-time.

As mentioned previously, \textit{in vitro} studies of ESC differentiation will set the groundwork for studying differentiation \textit{in vivo}. ESC cultures can be complex, where both ESC self-renewal and pluripotency are highly dependent upon feeder layers (FLs) and growth factors. FLs are composed of cells where DNA replication has ceased, while RNA and protein synthesis continues, hence the cells are mitotically inactive but remain viable. There are many drawbacks to current methods for growth-arresting FLs, thus, in the last part of this dissertation we aimed to develop a new tool for a successful long-term culture of undifferentiated ESCs. There are several criteria we had to meet in order for our tool to be successful: (i) the FL cells must be viable and mitotically inactivated; (ii) the FL must be able to sustain ESCs in their pluripotent state over several
subcultures; (iii) the ESCs must be able to differentiate into specific cell-lineages when incubated with an inducer; and (iv) the tool must be able to growth-arrest feeder cells from different species (e.g., zebrafish, mouse). Thus, this new technique offers an effective method for the successful long-term culture of pluripotent ESCs which is required for future in vivo mechanism studies and the development of new cell-based therapies.

This dissertation includes seven chapters. In Chapter I, we provide a brief overview of the research background and significance of this dissertation research, and outline the contents of each chapter.

In Chapter II, we describe the synthesis and characterization of stable and highly-purified photostable Au NPs, and developed DFOMS-MSIS to probe their transport into zebrafish embryos and to study their effects on embryonic development in real-time. We found that single Au NPs (11.6 ± 0.9 nm) passively diffused into the chorionic space of the embryos and continued their random-walk through the chorionic space and into the inner embryonic mass. Diffusion coefficients vary dramatically as NPs travel through various parts of the embryos, suggesting highly diverse transport barriers and viscosity gradients in the embryos. Furthermore, the amount of Au NPs accumulated in the embryos was concentration-dependent. However, their effects on embryonic development show random dependence on concentration. Majority of embryos incubated chronically with Au NPs for 120 h developed to normal zebrafish, with some being dead and few deformed. Au NPs were found in various parts of normally developed zebrafish, suggesting that random diffusion of NPs in embryos during their development might have led to stochastic effects on embryonic development, as illustrated in Figure 1. These results show that Au NPs are much more biocompatible than the same size Ag NPs that was reported elsewhere. The results provide powerful evidences that biocompatibility and toxicity of nanomaterials highly depend on their chemical properties, and the embryos can serve as effective in vivo nanotoxicology assays. Furthermore, these results suggest that Au NPs are a better suited biocompatible probe to be
Figure 1. Illustration of random diffusion of 12 nm Au NPs in zebrafish embryos leading to uncertain effects on embryonic development.

We synthesized and characterized 12 nm Au NPs and used them to probe the transport and diffusion into living zebrafish embryos and study their effects on embryonic development. We found that the majority of the Au NPs exhibit plasmonic green with some being orange in color, and that the Au NPs passively diffused into the embryos and caused very little death and deformity, with the majority of the embryos normally developed.
utilized for single NP imaging of embryonic development in real-time.

In Chapter III, we present the synthesis and characterization of stable and purified Au NPs (86.2 ± 10.8 nm), and further developed our dark-field single NP plasmonic microscopy and spectroscopy to study their transport into early developing zebrafish embryos and effects on embryonic development in real-time at single NP resolution. We found that single Au NPs (75 - 97 nm) passively diffused into the embryos via their chorionic pore canals, and stayed inside the embryos throughout their entire development (120 h). Similar to the smaller sized Au NPs, the diffusion coefficients of single NPs varied, further suggesting highly heterogeneous embryonic environments. The majority of embryos that were chronically incubated with the Au NPs for 120 h, developed to normal zebrafish, while an insignificant amount of embryos developed to deformed zebrafish or became dead, as illustrated in Figure 2. Interestingly, we did not observe dose-dependent effects of the Au NPs on embryonic development. By comparing with our previous studies of smaller Au NPs and similar sized Ag NPs, we found that the larger Au NPs are more biocompatible than the smaller Au NPs, while the similar sized Ag NPs are much more toxic than Au NPs. Thus, using zebrafish as an in vivo nanotoxicology assay we have shown the size- and chemical-dependence of Au and Ag NPs on embryonic development. Additionally, this study offers single NP microscopy and spectroscopy to characterize the biocompatibility and toxicity of single NPs, and new insights into the transport and toxicity of Au NPs in vivo in real-time, which are essential to the rational design of biocompatible NPs to be used as optical molecular probes and sensors for real-time in vivo imaging for a wide variety of biomedical applications.
Figure 2. Illustration of random diffusion of 86 nm Au NPs in zebrafish embryos leading to majority of embryos developed normally, with an insignificant amount of death and deformity.

We synthesized and characterized 86 nm Au NPs and used them to study their transport and diffusion into living zebrafish embryos and study their effects on embryonic development in real-time. We found that the majority of the Au NPs exhibit plasmonic green with some being orange and red in color, and were able to passively diffuse into the embryos and caused the majority of the embryos to develop normally with a negligible amount of death and deformity.
In Chapter IV, we describe the nanotoxicity of stable and purified Ag NPs (97 ± 13 nm in diameter) upon the specific developmental stages of zebrafish embryos using single NP plasmonic spectroscopy. We find that the Ag NPs induce specific developmental stage embryonic phenotypes and nanotoxicity in a dose-dependent manner, upon acute exposure, 2 h, to a given stage of embryos. As illustrated in Figure 3, we show that by determining the critical concentrations of the NPs that cause 50% of embryos to develop normally for cleavage, early gastrula, early segmentation, late segmentation and hatching stage zebrafish, that the earlier developmental stage embryos (i.e., cleavage stage) are much more sensitive to the effects of the NPs than the later stages (i.e., hatching stage). Interestingly, distinctive phenotypes are observed only in cleavage and early gastrula stage embryos treated with the NPs, showing the stage-specific effects of the NPs. In embryonic development, organ structures are not yet developed during the cleavage and early gastrula stages, thus, suggesting that the earliest determinative events to create these organs are ongoing, and disrupted by NPs, which leads to the downstream effects. By comparing with our previous studies of the smaller Ag NPs, we found that the embryonic phenotypes markedly depend upon the sizes of Ag NPs and embryonic developmental stages. These significant findings suggest that the Ag NPs can create distinctive mutants for further identification of biomarkers for better understanding of embryonic development and for potential diagnosis of birth defects. This study offers new information on how Ag NPs can be designed to achieve target specific studies and therapies for early embryonic development.
Critical concentration of the NPs

Figure 3. Illustration of probing stage-dependent biocompatibility and toxicity of 97 nm Ag NPs in living zebrafish embryos in real-time.

We synthesized and characterized stable (non-aggregated) and purified 97 nm Ag NPs and used them to study their effects on five different developmental stage of embryonic development. We incubated cleavage (2 hpf: hours post-fertilization), early gastrula (6 hpf), early segmentation (12 hpf), late segmentation (21 hpf) and hatching (48 hpf) stage embryos to the Ag NPs for 2 h and found that the earlier stages of development are much more sensitive to the Ag NPs than the later stages.
In Chapter V, we describe the development of a new, simple, green, and effective technique for ESC culture. As mentioned previously, growth-arrested feeder cells are essential to the culture and sustaining of undifferentiated ESCs, and are currently prepared using γ-irradiation or chemical inactivation. Both of these techniques have severe limitations. In this study, as illustrated in Figure 4, we utilized ultrashort pulsed electric fields (PEFs) to generate viable growth-arrested cells and used them as feeder cells for culturing and sustaining undifferentiated zebrafish ESCs over time. The PEFs can be generated using either a 10 nanosecond electric pulse (10nsEP) generator or 50 microsecond electric pulses (50μsEPs) using a widely available inexpensive conventional electroporator. We exposed rainbow trout spleen cells to 25 sequential 10nsEPs or 2 sequential 50μsEPs at varying electric fields. We found that cellular effects of the PEFs depended directly upon the pulse duration, number of pulses and electric field strength of the pulses, showing the possibility of tuning them to produce various types of growth-arrested cells for culturing undifferentiated ESCs. After exposure to a specific electric field, the 10nsEPs and 50μsEPs, generated high-quality growth-arrested feeder cells for proliferation of undifferentiated zebrafish ESCs over time. One can now use our new tool, PEFs, to prepare growth-arrested feeder cells for advancing ESC research and the possibility of using PEFs to replace conventional methods.
Figure 4. Illustration of cells exposed to ultrashort PEFs to produce viable growth-arrested feeder cells to culture and sustain undifferentiated ESCs.

We exposed zebrafish feeder cells, rainbow trout spleen cells, to ultrashort PEFs to generate viable growth-arrested feeder cells. We found that when exposed to the PEFs, the feeder cells were able to sustain zebrafish ESCs and keep them from differentiating.
In Chapter VI, we further developed and characterized our new tool designed in Chapter V. We previously described the use of PEFs to mitotically inactivate feeder cells, rainbow trout spleen cells, for the successful culture of zebrafish ESCs. In this study, as illustrated in Figure 5, we utilized these PEFs to generate growth-arrested feeder cells for the successful culture of undifferentiated mouse ESCs (mESCs). We found that the mouse embryonic fibroblast cells not only remained growth-arrested but were viable and metabolically active when exposed to 2 sequential 50μsEPs with a 5-s pulse interval at a specific electric field. Furthermore, over time the morphology and DNA content in the cells that were exposed to a specific PEF were comparable to cells exposed to γ-irradiation, which is the conventional method for preparing growth-arrested feeder layers and are commercially available. Additionally, when mESCs were seeded on feeder cells exposed to PEFs, they remained undifferentiated through several subcultures, as indicated by the positive expression of pluripotent ESC surface markers. Furthermore, when removed from the FLs and incubated with an inducer, the ESCs were able to differentiate into functional cardiomyocytes, demonstrating the FLs provided the necessary signaling molecules required for ESC differentiation. Notably, the mouse embryonic fibroblasts used as a FL for mESCs are also the same type of FL cells used for human ESC culture. Thus, PEFs can be used for the culture and expansion of human ESCs as well. These results illustrate that our new tool, PEFs, not only provide a cheaper and effective technique to prepare feeder cells for a successful large scale culture of pluripotent ESCs, but can be used with different species of ESCs.

In Chapter VII, we summarized our research findings described in the preceding chapters, emphasized the importance of this research and discussed the possible future research directions.
We exposed mouse embryonic fibroblast cells to PEF to produce viable and growth-arrested feeder cells for culturing mouse ESCs. We found that the feeder cells were metabolically and morphologically similar to those exposed to γ-irradiation. Additionally, mESCs remained undifferentiated through several subcultures and we were able to direct differentiation into functional cardiomyocytes.

Figure 5. Illustration of undifferentiated ESCs cultured on feeder cells exposed to PEFs or γ-irradiation and were able to differentiate into beating cardiomyocytes.
CHAPTER II

REAL-TIME IMAGING TOOLS FOR STUDY OF BIOCOMPATIBILITY AND TOXICITY OF 12 NM GOLD NANOPARTICLES IN SINGLE ZEBRAFISH EMBRYOS

INTRODUCTION

Au NPs have been used for a variety of applications for over 400 years.\textsuperscript{7} Since Au possesses inert chemical properties, it has been widely considered as one of most stable and biocompatible materials. Therefore, Au NPs have been suggested as potential biocompatible probes for living cellular imaging and as target-specific vehicles for drug delivery.\textsuperscript{1-5, 8, 10, 39-41} However, studies of biocompatibility and toxicity of Au NPs in various types of cells yield inconclusive results: some studies show toxic effects and high-dependence of toxicity on sizes and surface functional groups, while other studies exhibit no significant cytotoxicity.\textsuperscript{8, 41-51} Most of these studies did not use purified Au NPs, or examine any other chemicals present in Au NP solutions, or well characterize physical properties (e.g., possible size change and aggregation) of Au NPs in buffer solution and cell culture medium during the experiments, leading to inconclusive results.\textsuperscript{8, 41-51} Furthermore, study of biocompatibility and toxicity of Au NPs in living animals is still not yet fully explored.\textsuperscript{28, 48}

As noble metal NPs, Au NPs show unique optical properties such as localized surface plasmon resonance (LSPR), which is highly dependent upon their size, shape and surrounding environments.\textsuperscript{12-19} Single Au NPs exhibit strong Rayleigh scattering, offering the possibility of being directly observed and characterized using dark-field optical microscopy and spectroscopy (DFOMS) in real-time.\textsuperscript{10, 20-22} Their size-dependent LSPR spectra of single NPs allow us to characterize the sizes of individual NPs \textit{in vivo} (in living animals) in real-time using single NP optics, showing a great promise for imaging \textit{in vivo}.\textsuperscript{20, 22, 52-60}
Nonetheless, it is essential to determine their potential toxicological effects in vivo, prior to fully using them in living organisms.

Unfortunately, current nanotoxicity studies are carried out using a wide variety of cell lines and different types of living organisms, leading to impossible comparison of results between different studies, and inconclusive and conflicting reports. Conventional cytotoxicity assays are typically designed for ensemble measurements, which are inadequate for assessing the toxicity of NPs at the single-cell level, because NPs are quite often unevenly distributed among individual cells, demanding the study of nanotoxicity at single-cell resolution. More importantly, NPs are very different from conventional chemicals and drugs, and require special care to prevent them from aggregating in situ, and require new tools to characterize individual NPs in situ in real-time. Regrettably, in many nanotoxicity studies, such required cares were not taken to prevent aggregation of NPs during the experimental duration, and adequate tools were not developed and used to characterize stability (non-aggregation) and sizes of NPs in situ in real-time.

To overcome the limitations of current nanotoxicity studies, we have developed: (i) new methods to prepare stable (non-aggregated) and purified model NPs (e.g., different sizes and surface functional groups of Au and Ag NPs); (ii) real-time imaging tools (e.g., DFOMS) for characterizing the size of individual NPs in vivo in real-time; and (iii) effective in vivo assays (zebrafish embryos) for screening and probing the biocompatibility and toxicity of our model nanoparticles, aiming to depict the dependence of biocompatibility and toxicity of NPs on their physical and chemical properties, and their underlying mechanisms.

In our previous study, we used early development (cleavage stage) zebrafish embryos to study the transport, dose-dependent biocompatibility and toxicity of purified Ag NPs (11.6 ± 3.5 nm in diameter). In this study, we select the same cleavage stage zebrafish embryos as an in vivo model system to study
the transport mechanism and dose-dependent biocompatibility of the same size of purified Au NPs, aiming to investigate the dependence of biocompatibility and toxicity of NPs on their chemical properties. Study of the transport and effect of NPs on embryonic development can also provide new insights into how NPs affect embryonic development and offer new opportunities to develop biocompatible NP tools for a variety of applications, including in vivo imaging and drug delivery.

Zebrafish (Danio rerio) have been used extensively as a vertebrate model for embryological development study because of its small size, short breeding cycle, and wealthy genetic data base. Zebrafish embryos are transparent throughout every developmental stage and develop outside their mothers, allowing direct observation of the development of all internal organs without disturbing the embryos. The embryonic development is rapid: the first stages of development are completed in the first 24 hours post-fertilization (hpf); the normal embryo hatches by 72 hpf and fully develops at 120 hpf. Zebrafish can spawn large numbers of embryos, which can serve as effective and inexpensive in vivo assays for screening of biocompatibility and toxicity of NPs. Primary developmental mutations identified in zebrafish have close counterparts in other vertebrates, suggesting that zebrafish can be used effectively as a model for better understanding the developmental processes of higher organisms, including humans. Zebrafish represents an important aquatic species. Therefore, zebrafish embryos can serve as an important model system for us to investigate effects of nanomaterials on embryonic development, and assess their potential impacts on aquatic ecosystems and any possible environmental consequences. These unique features allowed us to probe transport and diffusion of single Au NPs inside the embryo and study their effects on the embryonic development in real-time, and use them as effective in vivo assays for screening the biocompatibility and toxicity of NPs and for possible monitoring of their potential environmental impacts.
RESULTS AND DISCUSSION

Synthesis and characterization of Au NPs

We synthesized Au NPs by reducing HAuCl₄ with sodium citrate. We carefully washed the Au NPs to remove any chemicals from the synthesis using centrifugation, and prepared highly purified and stable (non-aggregated) Au NPs. We determined concentrations, sizes and optical properties of Au NPs through each washing step. We further characterized the concentrations, sizes, and optical properties of the purified Au NPs dispersed in egg water (1.0 mM NaCl in DI water) for 120 h using UV-vis absorption spectroscopy, dynamic light scattering (DLS), high-resolution transmission electron microscopy (HR-TEM), and single NP dark-field optical microscopy and spectroscopy (DFOMS) (Figs. 6 and 7), aiming to determine their stability in egg water throughout the entire incubation period.

The absorbance spectra of freshly prepared and purified NPs before and after incubating with egg water for the entire experimental duration of 120 h (5 days) show an absorbance peak of 0.35 and a peak wavelength of 520 nm (FWHM = 53 nm) (Fig. 6A). This result indicates that the Au NPs are very stable in the egg water and remain non-aggregated over 120 h, suggesting that we can use the NPs to monitor the entire development of zebrafish embryos, which takes 120 h. We further characterized the sizes of the Au NPs using HR-TEM before and after incubation with egg water for 120 h, showing that the average sizes of Au NPs remain essentially the same at 11.6 ± 0.9 nm (Figs. 6B and C). We also measured the sizes of Au NPs in egg water over 120 h using DLS, showing that the sizes of Au NPs remain unchanged at 11.9 ± 1.6 nm. The NPs are hydrated in the solution, thus, their sizes measured by DLS appear to be slightly larger than those determined using HR-TEM.

We characterized the optical properties of individual Au NPs using DFOMS. The representative optical image of single Au NPs in Figure 7A shows that the majority of the Au NPs under a dark-field microscope are green, with few being orange. A color distribution of single Au NPs in Figure 7B illustrates that
Figure 6. Characterization of size and stability of Au NPs.

(A) UV-Vis absorption spectra of 1.2 nM Au NPs well-dispersed in egg water at 28°C for (a) 0 and (b) 120 h. The base-line subtracted absorbance was calculated by subtracting the base-line (dashed-line) from the peak absorbance using a well-known based-line subtraction method. The full width was then determined at the subtracted half-maximum. (B) HR-TEM images of Au NPs show the size and nearly spherical shape of single Au NPs. (C) Histogram of size distribution of Au NPs measured by HR-TEM show the average size to be 11.6 ± 0.9 nm. Scale bar = 20 nm.
88 ± 4% of NPs are green, with 12 ± 4% being orange. We determined the color distribution of single NPs in egg water over 120 h and found that they remained essentially unchanged over time, further demonstrating that single Au NPs are stable (non-aggregated) in egg water over time.

Representative LSPR spectra of single Au NPs acquired by DFOMS in Figure 7C show the peak wavelength of 565 nm (green color) (FWHM = 75 nm) and 600 nm (orange color) (FWHM = 59 nm), respectively. Single green Au NPs show lower scattering intensity than the orange NPs, suggesting that green ones are smaller than the orange ones. By comparing the histogram of color distribution of single NPs in Figure 7B with size distribution of single NPs determined by HR-TEM, we found that green NPs are correlated with 9 - 13 nm Au NPs while orange NPs are associated with 13.5 - 15 nm Au NPs.

As described by Mie theory, optical properties (LSPR) of noble metal NPs (e.g., Au, Ag) depend on their size, shape, surrounding environment, and dielectric constant of the embedding medium.13,14,19 As their spherical shape, surrounding environment, and dielectric constant of the embedding medium remain essentially in constant, peak wavelength of LSPR spectra of single NPs vary proportionally to the volume of single NPs, and scattering intensity of single spherical NPs are proportional to the sixth power of its radius.13,14 These unique optical properties allow us to use their color-index as size-index and intensity-index as size-index to determine sizes of single Au NPs in solution in real-time using dark-field optical microscopy, as we reported previously for the study of single Ag NPs.20-22,53-55

We further characterized the photostability of single Au NPs by acquiring sequential optical images of single Au NPs using DFOMS while these nanoparticles were constantly irradiated under a dark-field microscope illuminator (30 W halogen) over 12 h. The NPs were exposed to white-light illumination during the entire experiment. We measured the scattering intensity of individual
Figure 7. Characterization of the optical properties and photostability of single Au NPs.

(A) Representative dark-field optical image of single Au NPs shows that the majority of NPs are green with some being orange. Scale bar represents 2 μm, which shows the distance among individual NPs, but not the sizes of NPs, because they were imaged under optical diffraction limit. (B) Histogram of color distribution of individual Au NPs. (C) Representative LSPR spectra (color) of single Au NPs show peak wavelengths ($\lambda_{\text{max}}$) at 565 nm (green) (FWHM = 75 nm) and 600 nm (orange) (FWHM = 59 nm). (D) Plots of scattering intensity of (a) a representative single green Au NP and (b) background vs. illumination time.
NPs within a 20 x 20 pixel area (squared in Fig. 7A) and average background intensity of several detection areas with the same size of detection volume in the absence of NPs (dash-squared in Fig. 7A). We then subtracted the average background intensity from the integrated intensity of single NPs and individual background areas and plotted the subtracted integrated intensity of individual NPs and background as a function of time (Fig. 7D). These plots show that the scattering intensity of single Au NPs remains essentially unchanged over time. The slight fluctuations of scattering intensity of single NPs are similar to those observed from background, suggesting that these fluctuations might be attributable to intensity fluctuation of the microscope illuminator or to dark noise of the CCD camera. Therefore, these results demonstrate that single Au NPs are photostable and do not suffer photodecomposition and blinking, allowing us to use them as photostable probes to continuously probe the diffusion and transport dynamics throughout the early development of zebrafish embryos and investigate potential effects of single Au NPs on the embryonic developments.

**Diffusion and transport of single Au NPs in cleavage stage embryos**

To determine whether individual Au NPs can transport into living zebrafish embryos and their transport mechanism (active or passive), we incubated the purified Au NPs with the cleavage stage zebrafish embryos and imaged the diffusion and transport of single NPs into the cleavage stage embryos and inside the embryos in real-time.

The results in Figures 8 and 9 illustrates that single Au NPs (green and orange) can diffuse into embryos via chorionic pore canals on chorionic layers (CLs), through the chorion space (CS) and into the inner mass of embryos (IME). High-resolution black/white CCD images in Figures 8 and 9 show intra-embryonic structures in great detail, in which single Au NPs exhibit higher scattering intensities (much brighter) than any debris of embryos. We further characterized the LSPR spectra of single Au NPs observed in Figures 8 and 9 using DFOMS, which showed that the spectra of individual NPs are similar to those in Figure 7C.
The unique LSPR spectra of single Au NPs allow us to distinguish them from any possible tissue debris or vesicle-like particles in embryos, because unlike Au NPs, tissue debris do not possess plasmonic resonance and do not exhibit LSPR spectra (colors).

An optical image of a cleavage stage embryo in Figure 8A shows chorion, CS, yolk sac (YS), and IME. We focus on probing the transport of single Au NPs at the interface of the chorion with egg water (chorionic layers) and at the interface of the IME with the CS as squared by (B - C) in Figure 8A, respectively. Sequential dark-field optical images of chorionic layers in Figure 8B illustrate the transport of single Au NPs, as circled, from the egg water into the CS via chorionic pore canals. We clearly visualized arrays of well-organized chorionic pore canals (as squared in Fig. 8B: a) on chorionic layers and found each pore ranges 0.5 - 0.7 μm in diameter, and is about 1.5 - 2.5 μm apart. This result agrees well with what we reported previously and as determined by TEM.53, 72 Sequential dark-field optical images of the interface of CS with the IME show the transport of single Au NPs (circle) from the CS into the IME (Fig. 8C).

To further determine their transport mechanisms (active or passive), we investigate each diffusion trajectory of single NPs in egg water, entry into the CS, in the CS, and further into the IME (Fig. 9A) using the concept of 2D mean-square-displacement (MSD) and diffusion models (e.g., directed, simple and stationary Brownian diffusion).73-75 We tracked the diffusion of single NPs in various parts of embryos in real-time using real-time square-displacement (RTSD) (diffusion distance), instead of average (mean) square-displacement, because viscosity gradients and various transport barriers in embryos can vary diffusion coefficients of single NPs in embryos.53, 54 We use this approach to probe the diffusion of single NPs, transport barriers and viscosity of the different parts of embryos (e.g., CL, CS, IME) in real-time, and calculate the diffusion coefficient (D) of single NPs in simple Brownian motion by dividing the slope of a linear plot of square-displacement vs. time by 4 (Note: RTSD = 4DΔt) (Fig. 9B).
Figure 8. Real-time imaging of the diffusion and transport of single Au NPs in a cleavage stage zebrafish embryo.

(A) Optical image of the cleavage stage embryo shows chorion, CS, YS, and IME, acquired by CCD camera. The transport of single Au NPs at the interface of the chorion with egg water and at the interface of the CS with the IME is illustrated in (B - C), respectively. (B) Sequential dark-field optical images of the CL illustrate the transport of single Au NPs (circle), from the egg water into the CS via chorionic pore canals (square). The straight dashed lines outline the CL. The time interval between: (a) and (b) is 2.75 s; (b) and (c) is 3.92 s; and (c) and (d) is 5.10 s. (C) Sequential dark-field optical images of the interface of the CS with the IME illustrate the transport of single Au NPs (circle). The dotted lines outline the interface of the CS and the IME. The time interval between: (a) and (b) is 5.88 s; (b) and (c) is 5.89 s; and (c) and (d) is 3.14 s. Scale bars = 200 µm in (A), 10 µm in (B) and 20 µm in (C).
Representative diffusion trajectories of individual Au NPs with an identical orange color (size) show (Fig. 9A): (a) a single Au NP is diffusing into the embryo via chorionic pores at the exterior of a CL; (b) a single Au NP is diffusing into the IME from the CS; and (c) a single Au NP is diffusing in the CS.

Plots of RTSD of these single Au NPs vs. time (Fig. 9B: a) illustrate distinctive diffusion patterns of single Au NPs in various parts of the embryo. For instance, we found a restricted diffusion pattern (stationary Brownian diffusion), as the single Au NP diffused into the CS of the embryo from egg water via chorionic pores (Fig. 9B: a - i), suggesting that the single Au NP navigated through the pores and was tangled and trapped in the pores from time to time. The duration of single Au NPs passing through the pores in CLs range from 0.8 to 10 s. The diffusion coefficients of single NPs vary as it diffuses from egg water to the CLs, passing through the chorionic pores, and into the CS, ranging from $2.9 \times 10^{-11}$ to $1.0 \times 10^{-8}$ cm$^2$ s$^{-1}$ with an average of $2.3 \pm 1.9 \times 10^{-9}$ cm$^2$ s$^{-1}$.

In contrast, the diffusion of a single Au NP into the IME from the CS shows much less degree of restricted diffusion pattern and the NP diffuses freely in simple random Brownian motion (Fig. 9B: a - ii), suggesting that transport from the CS into the IME is less restricted than the CLs. These results imply that the transport barrier at the CL is higher than at the interface of the IME and CS, suggesting a much better protected surface (CL) of the CS than the IME. The diffusion coefficients of single NPs vary as it diffuses to and through the interface of CS and IME, ranging from $1.0 \times 10^{-9}$ to $9.5 \times 10^{-9}$ cm$^2$ s$^{-1}$ with an average of $3.3 \pm 2.4 \times 10^{-9}$ cm$^2$ s$^{-1}$. Interestingly, diffusion of single Au NPs in the CS exhibits simple random Brownian motion with uneven diffusion coefficients, ranging from $3.9 \times 10^{-10}$ to $1.3 \times 10^{-8}$ cm$^2$ s$^{-1}$ with an average of $4.9 \pm 0.8 \times 10^{-9}$ cm$^2$ s$^{-1}$. This result suggests a highly heterogeneous CS with various viscosities, which is similar to what we reported previously.

The results show that Au NPs can diffuse into every part of the embryos from egg water via passive Brownian diffusion (no active transport mechanism),
Figure 9. Characterization of transport and diffusion trajectories of single Au NPs in a cleavage stage embryo.

(A) Diffusion trajectories of an orange Au NP at (a) the exterior surface of the CL, (b) at the interface of the CS with the IME, and (c) in the CS. (B) Plots of RTSD of single Au NPs as a function of time: (a) from the diffusion trajectories (a – c) shown in (A), illustrating that the orange Au NP shows (i) restricted diffusion, with $D = 2.3 \pm 1.9 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ at the exterior surface of CLs; (ii) partially restricted diffusion, with $D = 3.3 \pm 2.4 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ at the interface of the CS and the IME; and (iii) random Brownian motion, with $D = 4.9 \pm 0.8 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ in the CS, respectively. (b) From representative (i) orange and (ii) green Au NPs in egg water. Both NPs display random Brownian motion with $D = 1.3 \pm 1.0 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ and $2.7 \pm 2.5 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$, respectively.
because the plots of RTSD vs. time in Fig. 9B show the same characteristics as those described in diffusion models for simple passive Brownian diffusion and stationary Brownian diffusion, but not for directed (active) Brownian diffusion.73-75

Thus, individual Au NPs are not transported into embryos from egg water or inside embryos via active transport pathways (e.g., uptake of NPs driven by the energy of living embryos).

We also characterized the diffusion models and diffusion coefficients of single green and orange Au NPs in egg water (Fig. 9B: b) to determine the possible variation of diffusion coefficient of individual NPs due to the slight variation of their sizes, which serves as a control experiment to determine the variation of diffusion coefficients among single NPs. We found that single orange and green Au NPs show simple Brownian diffusion in egg water with $D$ of $1.3 \pm 1.0 \times 10^{-8}$ and $2.7 \pm 2.5 \times 10^{-8}$ cm$^2$ s$^{-1}$, respectively. It is well known that diffusion coefficients of single NPs are inversely proportional to their radius, as described by the Stoke-Einstein equation, $D = kT/(6\pi\eta a)$, which shows that the diffusion coefficient ($D$) depends on the viscosity of medium ($\eta$) and the radius ($a$) of solute (NP).76-78 As we described previously in Figures 6 and 7, the Au NPs with LSPR spectra (colors) at green and orange region are correlated with NPs with 9 - 13 nm and 13.5 - 15 nm in diameter, respectively. Thus, the diffusion coefficient of the larger NPs (15 nm) is about twice smaller than that of the smaller NPs (9 nm), as shown in Figure 9B: b. Note that the shapes of nanoparticles are not perfectly spherical. Thus, the aspect ratios of individual nanoparticles vary, which also contribute to the small variation in their diffusion coefficients.

We studied the diffusion coefficients of same color (radius) of Au NPs in embryos and compared with those in egg water, showing that the various diffusion coefficients observed in three different parts of embryos (Fig. 9B: a) are indeed attributable to the viscosity gradient and transport barrier of embryos, but not the different size of individual NPs. The diffusion coefficients of single Au
NPs inside the chorionic space (Fig. 9B: a - iii) are about 2 - 110 times smaller than those NPs with identical orange color (size) in egg water (Fig. 9B: b - i), showing a wide variation of the viscosity gradients in CS.

**Imaging and characterization of individual Au NPs in embryos**

We imaged and characterized NPs accumulated and embedded in living embryos using DFOMS, during the incubation of cleavage stage embryos with 1.2 nM Au NPs for 4 h, during which the embryos developed from the cleavage stage to gastrulation stage (Fig. 10). We observed green and orange Au NPs in various parts of the embryos, (circle in Fig. 10A), which shows that single Au NPs are embedded in the pores of CLs (Fig. 10A: b - c), inside CS (Fig. 10A: d) and IME (on the surface of embryonic cells in Fig. 10A: e). We found that the LSPR spectra of these embedded single NPs are similar to those observed in Figure 7C, showing that they were indeed Au NPs. Notably, the cleavage stage and gastrulation stage embryos have not yet developed any pigmentation, and any cellular and tissue debris of embryos appear white under dark-field microscope and do not show signature LSPR spectra (colors) of single Au NPs. Thus, the unique feature of LSPR spectra of single Au NPs allows us to distinguish them from any possible tissue debris or vesicle-like particles in embryos.

We found that majority of NPs were transported into the CS and some of them were overlapped with chorionic pore canals (Fig. 10A: b - c). The representative LSPR spectra of individual NPs in the CS (Fig. 10A: b) show the similar peak wavelength as those observed in egg water in Figure 8C. This result indicates that majority of NPs remain non-aggregated inside embryos. Otherwise, we would have observed a significant red shift in the LSPR spectra of individual NPs. Note that the images in Figure 10A were acquired in a whole living embryo in real-time. Since the entire embryo was filled with NPs, it led to a high background in the color images (Fig. 10A: b - e).

We also incubated cleavage stage embryos with various concentrations of Au NPs, and imaged Au NPs accumulated in embryos, aiming to determine
the concentration-dependence of accumulated Au NPs in embryos. The images in Figure 10B clearly show that the colors of embryos (6 hpf) depend on the incubation concentration of Au NPs, showing colorless of embryos in egg water (the absence of Au NPs), the light-red and dark-burgundy (color of Au NPs in solution) as cleavage stage embryos are incubated with 0, 0.05, 0.20, and 1.2 nM Au NPs for only 4 h. This result indicates that the amount of Au NPs accumulated in embryos increases as Au NPs concentration increases, suggesting that concentration-gradient of Au NPs may be the driving force for transport of Au NPs into the embryos, which agrees well with entry of single nanoparticles into embryos via passive diffusion of random Brownian motion as shown in Figures 8 - 9.

Effects of Au NPs on embryonic development

To probe the possible concentration-dependence of the effects of Au NPs on embryonic development, we incubated cleavage stage embryos with a series of washed (purified) Au NP solutions (0, 0.025, 0.05, 0.10, 0.20, 0.40, 0.60, 0.80, 1.0, 1.2 nM) for 120 h, as they fully developed to zebrafish. We imaged the embryos throughout their developmental stages and characterized live, dead and deformed zebrafish as they fully developed at 120 h. Plots of percentages of live, dead and deformed zebrafish vs. concentration of Au NPs in Figure 11A illustrate that in the absence of NPs (control experiment), 82 ± 10% of zebrafish were alive and normally developed, while 18 ± 10% of zebrafish were dead, but no deformed zebrafish were observed.

As the Au NP concentration increases from 0.025 to 1.2 nM, the percentages of alive and normally developed zebrafish decrease slightly within the margin of errors, ranging from 81 ± 13% to 63 ± 20%, and the percentages of dead zebrafish increase from 17 ± 11% to 31 ± 18%. Interestingly, the percentage of deformed zebrafish ranges from 0 to 6.6 ± 5.5% in the presence of Au NPs, showing stochastic dependence of NP concentration.
Figure 10. Characterization of Au NPs in zebrafish embryos using DFOMS. (A) Representative optical image of (a) a cleavage stage embryo shows single Au NPs with multiple colors in (b) the intra- and (c) extra-surface of CL, illustrating well-organized chorionic pores; (d) in CS and (e) in the IME, as squared in (a). A few representative NPs embedded in the tissues of interest are circled in (b – e) to help the identification of NPs. (B) Optical images of the gastrulation stage embryos in (a) egg water alone and incubated with (b) 0.05, (c) 0.20, and (d) 1.2 nM Au NPs for 4 h since its cleavage stage. The embryos in (b – d) show the light-red and dark-burgundy color of Au NPs, suggesting that the amount of accumulated Au NPs in the embryos increases with concentration. Scale bars = 200 µm in (a), 20 µm in (b – c), 10 µm in (d), 1 µm in (e) and 500 µm in (B).
To ensure that the deformed zebrafish is not caused by any possible residual chemicals from synthesis of NPs we performed negative control experiments by replacing Au NPs with equal amount of supernatants which was generated from washing NPs. The result in Figure 11B illustrates that the percentages of normally developed live zebrafish and dead zebrafish are essentially same as those observed in the absence of Au NPs in Figure 11A, and deformed zebrafish was not observed in 0.0, 0.10, 0.60 and 1.2 nM supernatants. Taken together, deformed zebrafish were observed only in the embryos treated by Au NPs, but not in any control experiments (e.g., egg water or supernatants), indicating the toxicity of Au NPs.

We compare normally developed zebrafish (Fig. 12A) with abnormally developed zebrafish (Fig. 12B) to identify the types of deformities. We found a wide variety of deformities, including finfold (FF) abnormality, tail and spinal cord flexure and truncation (TF), cardiac malformation (CM), yolk sac edema (YSE), and acephaly (headless) and no tail (NT/A), as illustrated in Figure 12B.

In normally developed zebrafish, the median finfold is a clear, thin membrane around the entire trunk region containing unsegmented fin rays, and the notochord and spinal cord develop straight to the posterior-most tip of the tail (Fig. 12A: a - b). In contrast, the tissue structure of the finfold of zebrafish is disorganized and the shapes of the finfold and fin rays are altered (Fig. 12B: a - b), which is classified as a finfold abnormality. The finfold abnormality is often accompanied by tail and spinal cord flexure and truncation (Fig. 12B: a - d). A few embryos exposed to Au NPs also display a form of cardiac malformation in fully developed zebrafish, where the pericardial sac region is extremely large and the cardiac ventricle is shrunk (Fig. 12B: e - f). The yolk sac region is a bulbous area containing yolk that provides nutrients to the developing embryo, and it shrinks during the later developmental stages in normally developed zebrafish (Fig. 12A: c). In contrast, the yolk sac region of zebrafish is swollen and enlarged (Fig. 12B: e - f), which is classified as yolk sac edema.
Figure 11. Effect of Au NPs on embryonic development.

(A) Histogram of the distribution of normally developed (blue), dead (red), and deformed (pink) zebrafish vs. concentration of Au NPs; (B) Histogram of the distribution of normally developed (blue) and dead (red) vs. concentration of supernatant removed from washing Au NPs (negative control).
The most severe and rare deformation of zebrafish that we have observed in this study is acephaly (absence of a developed head) and the lack of a distinct tail region (Fig. 12B: g), while the heart of such a deformed zebrafish is still beating. This severely deformed zebrafish shows a small amount of head-like tissue where the head would normally develop, and the tissue was not a fully developed head but rather an irregular formed mass of tissue. Notably, we found only one out of more than 1000 embryos treated with Au NPs developed this acephaly deformation. Typically, multiple types of deformations are observed in the same zebrafish (Fig. 12B), suggesting that some of these deformations may be inter-related.

To determine possible dependence of types of deformity (e.g., finfold abnormality, tail and spinal cord flexure and truncation, cardiac malformation, yolk sac edema, acephaly and no tail) on NP concentration, we plotted the number of given types of deformed zebrafish vs. NP concentration (Fig. 12C), showing the highest number of deformed zebrafish and most types of deformities at 0.20 nM. To determine any possible dependence of a specific type of deformity on NP concentration, we plotted the percentages of given types of deformed zebrafish (= number of one given type of deformed zebrafish divided by the total number of deformed zebrafish at a given concentration) vs. NP concentration (Fig. 12D). We found that the percentage of any given type of deformed zebrafish varies with NP concentration.

Taken together, the result in Figure 11 illustrate that cleavage stage embryos chronically treated with Au NPs for 120 h result in small numbers of dead and abnormally developed zebrafish. The treated embryos show no clear trend of increase in death or development of deformed zebrafish (types of deformity and severity), as NP concentration increases. By comparing this result with our previous observations of the effects of the same size and concentration of Ag NPs on the same stage of embryos, we find that Au NPs caused negligible amount of dead and deformed in comparison with Ag NPs, suggesting that Au NPs are much more biocompatible and less toxic to embryos.
Figure 12. Normal and deformed zebrafish resulting from chronic treatment with Au NPs since their cleavage stage.

(A) Representative optical images of normally developed zebrafish showing the normal development of the (a) finfold (FF); (b) tail/spinal cord; (c) cardiac and yolk sac; and (d) head and eyes. (B) Representative optical images of zebrafish at a given concentration of Au NPs showing several deformities such as (a – b) finfold abnormality (FF); (c – d) tail/spinal cord flexure and truncation (TF); (e – f) cardiac malformation (CM) and yolk sac edema (YSE); and (g) acephaly and no tail (NT/A). (C) Histograms of numbers of deformed zebrafish with each type or deformity vs. concentration of Au NPs. (D) Histograms of the percentages of deformed zebrafish with each type of deformity vs. concentration of Au NPs. Scale bars = 500 μm in (A), 250 μm in (B: a - b), 1000 μm in (B: c – f), and 500 μm in (B: g).
Nonetheless, the same types of deformed zebrafish were observed in both studies (treating embryos with either Au or Ag NPs). A plausible explanation for these interesting observations is that the random Brownian diffusion of Au NPs inside embryos during their development (120 h) might have led to the accumulation of various amounts of Au NPs in various parts of the embryos stochastically.

Imaging and characterization NPs in fully developed zebrafish

We imaged and characterized Au NPs embedded in fully developed zebrafish that had been chronically incubated with a given concentration (1.2 nM) of NPs for 120 h since their cleavage stage. We rinsed the zebrafish with DI water to remove external NPs, and fixed them using a histology protocol for tissue sample preparation. We prepared ultrathin-layer sections (~0.25 - 4 μm thickness) of the zebrafish by carefully sectioning the tissues of interest (e.g., eye retina, brain, nasal epithelium, otic capsule, stomach, notochord, swim bladder, and pectoral fin) using a microtome. Finally, we imaged and characterized LSPR spectra of individual NPs embedded in the tissues of interest using DFOMS (Fig. 13).

We observed green and orange Au NPs in various parts of the normally developed zebrafish. The results in Figure 13B show that Au NPs are embedded in (i) retina, (ii) brain tissue, (iii) nasal sensory epithelium, (iv) otic capsule, (v) stomach, (vi) notochord, (vii) swim bladder, and (viii) pectoral fin. To enhance the clarity of presentation, we did not circle all embedded NPs in the tissues, but highlighted a few representative NPs embedded in the tissues of interest, as those circled in Figure 13B. LSPR spectra of individual embedded NPs are similar to those observed in Figure 7C, showing that they are indeed Au NPs and majority of embedded NPs remain non-aggregated. If Au NPs were aggregated, the sizes of single NPs would become larger, which would lead to the large shift or peak wavelength of the LSPR spectra of single NPs and color changes of single NPs. We found that the entire section of tissue was fully embedded with
NPs. LSPR scattering of individual NPs lighted up their surrounding tissue, creating a high background, which was not observed in the tissues of zebrafish untreated with NPs. The unique feature of LSPR spectra of single Au NPs (Fig. 7C) allows us to distinguish them from any possible tissue debris, because tissue debris does not exhibit such LSPR spectra under dark-field microscopy.

In comparison with TEM, we found several unique advantages of using histology protocols to prepare tissue samples and using LSPR spectra to characterize embedded individual Au NPs in tissue samples. For example, we can align the zebrafish in the desired position and perform ultrathin-layer section of specific areas of zebrafish. We can characterize the tissue of interest with no need of using staining reagents. Note that staining reagents quite often create significant background and interfere with the identification of individual NPs. We can characterize a massive amount of tissue samples rapidly using DFOMS, allowing us to carry out extensive studies, generating massive amounts of data for statistical analysis, as well as to investigate rare events of interest. We can qualitatively characterize individual Au NPs and determine their sizes using their unique LSPR spectra. Note that LSPR spectra depend upon the size, shape and surrounding environments of NPs. By carefully calibrating the LSPR spectra of individual NPs vs. their sizes, as described above in Figures 6 – 7, we can determine the size of individual NPs at the nanometer scale using their LSPR spectra via DFOMS.

Although TEM has been widely used to characterize NPs embedded in cells and tissues, and TEM can offer much higher spatial resolution,\textsuperscript{8, 21, 48, 61} the protocols for TEM sample preparation are cumbersome, and it is nearly impossible to control the alignment of biological organisms (e.g., zebrafish and cells) in resin blocks. Thus, the sections of tissues and cells are generated with random locations of organisms, and it is hard to prepare reproducible sections of interest for comparison, to generate and characterize massive amounts of tissue sections for statistical analysis, and to probe rare events of interest. More problematically, to observe the characteristic locations (morphologies) of tissues
Figure 13. Characterization of Au NPs embedded in a fully developed zebrafish.

(A) Optical color images of a fixed (a) longitudinal section and (b) cross-section of a normally developed zebrafish. The rectangles highlight representative areas: (i) retina, (ii) brain tissue, (iii) nasal sensory epithelium, (iv) otic capsule, (v) stomach, (vi) notochord, (vii) swim bladder, and (viii) pectoral fin. (B) Zoomed-in optical images of those tissue sections outlined in (A). The circles highlight representative individual Au NPs embedded in the tissue sections. Scale bars = 200 µm in (A) and 4 µm in (B).
and cells using staining reagents (e.g., uranyl acetate and lead citrate), which quite often creates significant background and interferes with identification of NPs embedded in tissues. To definitively identify embedded NPs of interest, one needs to perform EDS of the embedded NPs in tissues and cells. Unfortunately, for NPs that are smaller than 15 nm, it is extremely challenging to obtain a sufficiently high signal from individual NPs over the background and noise of the surrounding tissues and those reagents used to prepare and fix the tissues and cells. To gain a sufficiently high EDS signal from individual NPs and reduce noise and background, one can increase the voltage of the electron beam and/or focus the electron beam on the NPs of interest (reduce the size of the focus spot to decrease the signal of surrounding tissues). However, current technologies provide micrometer-square areas of EDS scanning. Furthermore, tightening the beam area and using higher voltages for the electron beam leads to exposure of biological samples to higher energies, resulting in the evaporation of surrounding tissues and cells (burning a hole through tissue samples) and loss of signals of interest. These limitations may be the reasons why EDS characterization of embedded NPs (especially smaller NPs, <15 nm) in tissues and cells have rarely been reported, even though TEM images have been widely displayed in the literature.

Therefore, it is crucial to explore new and effective methods too quantitatively and qualitatively image and characterize embedded individual NPs in tissues and cells. We found that the methods that we have reported in this study are more powerful, informative, convenient, and much lower cost than current conventional TEM methods for imaging and characterization of individual NPs embedded in tissues.

By comparing the results in Figure 13 with those in Figure 10, we found that NPs that had diffused into the early developing embryos stayed inside the embryos during the entire development, which led to the NPs embedded in various parts of fully developed zebrafish. The most intriguing question here is what factor defines the developmental fate of the cleavage stage embryos to
become normally developed, deformed (various types of deformation), or dead zebrafish.

As we found in Figure 13, normally developed zebrafish are embedded with significant amount of Au NPs all over their bodies, and the results in Figures 11 and 12 illustrate that percentages of dead and deformed zebrafish and types of zebrafish deformation are not proportionally correlated with the concentration of Au NPs. These interesting findings further suggest what we speculated previously: that the random walk (random Brownian diffusion) of Au NPs inside embryos during their development (120 h) might lead to stochastic toxic effects on embryonic development. It is quite possible that various parts of embryos might have different responses to Au NPs. If the majority of the portions of the embryos are tolerant to Au NPs, it will be entirely stochastic and less concentration-dependent for individual Au NPs to randomly walk into the vital portion of embryos that is much more sensitive to Au NPs, leading to abnormal development of zebrafish. The other possible explanation is that some embryos may be more tolerant to Au NPs than others. Thus, some embryos develop normally, while others are dead or deformed.

SUMMARY

In summary, we have synthesized and characterized stable (photostable, non-aggregating), nearly monodisperse, and highly-purified Au NPs, and utilized them to study cleavage stage embryos in real-time and to probe their effects on embryonic development. We found that single Au NPs passively diffused into the chorionic space of the embryos via their chorionic pore canals and continued their random walk into inner mass of embryos. Diffusion coefficients of single NPs range from $2.8 \times 10^{-11}$ to $1.3 \times 10^{-8}$ cm$^2$ s$^{-1}$, as NPs passively diffuse through various parts of embryos, suggesting highly diverse transport barriers and viscosity gradients of the embryos. A wide range of diffusion coefficients ($3.9 \times 10^{-10}$ to $1.3 \times 10^{-8}$ cm$^2$ s$^{-1}$) of single NPs in the chorion space suggests its high
heterogeneity. We found that the amount of Au NPs accumulated in embryos increases with its concentration. Interestingly, their effects on embryonic development show stochastic dependence on concentration. The majority of embryos (74% on average) chronically incubated with 0.025 – 1.2 nM Au NPs for 120 h developed to normal zebrafish, with some (24%) being dead and a few (2%) deformed. This result is in stark contrast with what we reported previously for Ag NPs, showing that Au NPs are much more biocompatible with the embryos than Ag NPs and suggesting that the biocompatibility and toxicity of NPs depends on their chemical properties. We describe a new approach to image and characterize individual Au NPs embedded in tissues using histology sample preparation methods and LSPR spectra of single NPs. We found Au NPs in various parts of normally developed zebrafish. Taken together, these interesting findings suggest that the random walk (Brownian diffusion) of Au NPs in embryos during their development (120 h) causes individual Au NPs walking into different parts of embryos randomly, which might have led to stochastic effects on embryonic development.

METHODS

Reagents and supplies

Deionized (DI) water (18 MΩ, Barnstead) was used to prepare solutions and rinse glassware. All chemicals were purchased from Sigma and used without further purification or treatment.

Synthesis and characterization of Au NPs

We synthesized Au NPs by reducing a 1.0 mM aqueous solution of HAuCl₄•3H₂O with 38.8 mM sodium citrate solution. As we were refluxing, stirring and boiling the solution for 30 min, we found that the solution changed from yellow to colorless, then to dark purple and finally to burgundy. We stopped heating the solution, continued stirring for 30 min, and filtered the solution using a
0.22 μm filter. The NPs were then washed twice with deionized (DI) water using centrifugation (7735 relative centrifugal force (rcf), Beckman J-21) to remove the by-products of the reaction and any excess of chemicals in solution. The washed NPs in the pellets were then resuspended in DI water and used for incubation with embryos. The supernatant of the NP solution removed from the last washing step was collected and used as a negative control experiment to monitor the effects of possible trace chemicals involved in NP synthesis that might be present in the NP solution.

We characterized the concentrations, optical properties, and sizes of NPs using UV–vis spectroscopy (Hitachi U-2010), dark-field optical microscopy and spectroscopy (DFOMS), high-resolution transmission electron microscopy (HR-TEM) (FEI Tecnai G2 F30 S-Twin), and dynamic light scattering (DLS) (Nicomp 380ZLS).

We have described in detail, in our previous studies, the design and construction of our DFOMS (also called as SNOMS) for real-time imaging and characterization of LSPR spectra of single NPs in solution, in single living cells, in zebrafish embryos, and for single-molecule detection. In this study, a CoolSnap HQ2 CCD camera and EMCCD camera (PhotonMAX) coupled with a Spectrograph (SpectraPro-2150, Roper Scientific), and a color digital camera were used for imaging and characterization of LSPR spectra of single Au NPs.

**Analysis and characterization of molar concentrations of Au NP solutions**

We calculated the molar concentration of unwashed Au NPs as described previously. We also used DFOMS to determine any trace amount of individual Au NPs in the supernatants. If we found any Au NPs in the supernatant, we would further remove them using ultra-centrifugation (L90, Beckman), which allowed the supernatant to serve as a control experiment (all other chemicals, except Au NPs, are present in the solution). Note that we would continue washing cycles (three or four washing cycles) until we generated a supernatant
that did not affect the embryonic development (clean blank control experiments),
which indicated that the NPs were well purified and ready to be used to treat the
embryos. In this study and our previous studies,\textsuperscript{53} \textsuperscript{54} two-times washed
supernatants provided clean and successful control experiments. In other words,
the twice-washed NPs are sufficiently pure to be used to study their effects on
embryonic development.

**Characterization of photostability of single Au NPs**

We characterized photostability of single Au NPs by acquiring sequential
optical images of single Au NPs using EMCCD camera with exposure time at 200
ms and interval time of 40.6 ms for the first 5 min and 300 s afterwards while
these NPs were constantly irradiated under dark-field microscope illuminator (30
W halogen) for 12 h. The illumination power at the sample stage (focal plane of
dark field) measured using a power meter was $0.070 \pm 0.001$ W during the
experiment.\textsuperscript{52} \textsuperscript{54} \textsuperscript{56} We calculated the integrated intensity of a $20 \times 20$ pixel area
where a NP was presented (as squared in Fig. 7A) and the average background
intensity of several detection areas with the same size of detection volume ($20 \times
20$ pixel) in the absence of NPs (dashed square in Fig. 7A). By subtracting the
average background intensity from the integrated intensity of single NPs and
individual background area, we plotted the subtracted integrated intensity of
individual NPs and background as a function of time (Fig. 7D).

**Breeding of zebrafish embryos**

We housed wild-type adult zebrafish (Aquatic Ecosystems) in a stand-
alone system (Aquatic Habitats), maintained and bred zebrafish, as described
previously.\textsuperscript{53} \textsuperscript{54} \textsuperscript{86} Briefly, we placed two pairs of mature zebrafish into a clean
10-gallon breeding tank, and used a light (14 h) - dark (10 h) cycle to trigger
breeding and fertilization of embryos. We collected the embryos at cleavage
stage ($8 - 64$-cell stage; $0.75 - 2.25$ hpf), transferred them into a Petri dish
containing egg water ($1.0$ mM NaCl in DI water) (NaCl, 99.95%, Sigma), and well
rinsed them with egg water to remove the surrounding debris. All experiments
involving embryos and zebrafish were conducted in compliance with the IACUC guidelines (protocol #11-030).

**Study of transport and biocompatibility of Au NPs in embryos**

To probe the transport of Au NPs into embryos in real-time, we then placed the cleavage stage embryos directly into a home-made microchamber, and simultaneously imaged the transport of single NPs into embryos in real-time using our DFOMS, while adding purified Au NP solutions into the chamber to prepare desired Au NP concentrations. The diffusion measurements were completed within minutes.

To determine biocompatibility of Au NPs, we transferred the embryos into 24-well plates (two embryos per well) and incubated them with a series of washed (purified) Au NP solutions (0, 0.025, 0.05, 0.10, 0.20, 0.40, 0.60, 0.80, 1.0, 1.2 nM). We also incubated embryos with egg water (in the absence of NPs) and the supernatants from washing Au NPs, which were carried out simultaneously as those incubated with Au NPs, serving as control experiments for monitoring untreated embryos and determining possible effects of trace chemicals involved in synthesis of NPs, respectively. We incubated the embryos in the 24-well plates in a water bath at 28.5°C, and observed them at room temperature using an inverted microscope (Zeiss Axiovert 100) at 24, 48, 72, 96 and 120 hpf. We acquired images of the normal, deformed and dead zebrafish at 120 hpf using a digital camera and CCD camera. Each experiment was done at least four times and a total of 60 – 64 embryos for each experiment were studied to gain sufficient amount of statistics.

**Imaging and characterization of Au NPs in embryos and zebrafish**

We imaged and characterized Au NPs accumulated in living embryos using DFOMS as the cleavage stage embryos were incubated with 1.2 nM Au NPs for 4 h. We also characterized Au NPs embedded in fully developed zebrafish that had been chronically incubated with a given concentration (1.2 nM).
of NPs for 120 h since their cleavage stage. The treated zebrafish were rinsed with DI water to remove external NPs, and fixed using a tissue processor (STP 120) and a tissue embedding center (Shandon Histocentre™ 3 Embedding Center) via a histology protocol of tissue sample preparation as described below.

The zebrafish were fixed using chemical fixation (formaldehyde), dehydrated by EtOH, infiltrated with Clear-Rite (isoparaffinic aliphatic hydrocarbons), and finally embedded with paraffin, using a Microm STP-120 Spin Tissue Processor (Thermo Fisher Scientific). The tissue processor contains 12 buckets of solutions and a tissue sample holder that is controlled by computer programs to automatically move the tissue samples from a solution in one bucket to another in a desired manner. The solutions in the 12 buckets are arranged in the following order: buckets (i) and (ii): 10% buffered formalin for fixation; buckets (iii) – (viii): 50%, 70%, 95%, 100%, 100%, and 100% (v/v) of EtOH/water for dehydration, respectively; buckets (ix) and (x): Clear-Rite for removing EtOH from the tissue and infiltrating the tissue with Clear-Rite; buckets (xi) and (xii): paraffin at 60°C for embedding the tissue with paraffin. We placed the zebrafish treated with NPs (or supernatant or untreated, as control experiments) in histoscreen cassettes and transferred the cassettes to the sample holder of the tissue processor, which moved the samples from one bucket to the other, allowing the tissue of zebrafish to be fully immersed in the solution of each bucket for the desired duration (20–40 min) to complete the histology sample preparation.

For example, the zebrafish were first immersed in buckets (i) and (ii) for 20 min each to fully infiltrate the tissue of zebrafish with fixative. Note that formaldehyde reacts with the amine groups (–NH₂) of tissue proteins and stabilizes the tissue in a fixed position. Dehydration was then performed to remove water from the tissue of zebrafish by fully immersing the samples into each of the solutions in buckets (iii) – (viii), for 20 min each. The samples were then fully immersed in buckets (ix) – (x) for 20 min each, which allowed Clear-Rite (a solvent miscible with the embedding medium, paraffin) to completely replace the EtOH that remained inside the tissue. The samples were finally
moved into the last two buckets (xi – xii) and fully immersed in each paraffin
solution for 40 min each. The heat (60°C) causes the Clear-Rite solvent to
evaporate, creating spaces in the tissue of zebrafish, which were fully infiltrated
with the heated paraffin. Note that it is crucial to completely remove water from
the tissue by dehydration and fully infiltrate the tissue with Clear-Rite in order to
appropriately embed the tissue with paraffin and to prevent the formation of holes
in the tissue, allowing us to prepare ultra-thin-layer sections of tissue samples of
zebrafish in the following steps.

We then moved the zebrafish with the histo-screen cassettes from the
sample holder of tissue processor into a paraffin bath at 60°C in the embedding
center, and used the embedding center to prepare the tissue sample blocks. The
embedding center includes a paraffin bath at 60°C, a well-controlled nozzle
system of the paraffin bath, a hot plate at 45°C, and a cold plate at 0°C. We
added a thin layer of liquefied paraffin (60°C) to the bottom of a small histological
block mold using the nozzle system of the paraffin bath, removed one zebrafish
from the histo-screen cassette to the block mold, and aligned the zebrafish in the
desired position in the mold (either vertically or horizontally aligned with the
bottom of the mold). The mold was placed on the hot plate (45°C) to prevent the
paraffin from hardening, allowing us to perform the alignment successfully. We
then placed the mold on the top of the cold plate (0°C), which immediately
solidified the paraffin and locked the zebrafish in the desired position inside the
paraffin block. We filled up the mold with the paraffin (60°C), placed the histo-
screen cassette on the top of the mold, and left it on the cold plate (0°C)
overnight, allowing the paraffin to solidify completely.

We sectioned the sample block (~0.25 – 4 μm thickness) using a Microm
HM360 rotary microtome (Thermo Fisher Scientific), and floated each section of
the block in a DI water bath (40°C), allowing the section to well spread over the
water surface and create a smoother and flatter section. We then collected the
samples using specially designed tissue slides, and dried the slides on a slide
warmer (45°C) overnight. After the slides were dried, we heated the slides in an
upright position in an oven at 60°C for 30 min, allowing the paraffin to slowly melt off the slides to remove the excess paraffin from the tissue.

The sections of the tissue embedded with NPs were directly characterized using DFOMS (Fig. 13). LSPR spectra of individual NPs offer chemical characterization of the NPs (Fig. 7C). The methods that we have developed in this study and in our previous studies provide powerful new tools to determine and characterize individual NPs embedded in tissues, and to image the tissues with embedded NPs with no need of staining reagents.

**Statistical Analysis**

Each experiment related to the study of biocompatibility and toxicity of Au NPs was performed at least four times and a total of 60 - 64 embryos for each experiment were studied to gain sufficient data for statistical analysis, permitting the study of effect of NPs on a bulk amount of embryos at the single-embryo level. We present average percentages of normally developed, dead and deformed zebrafish vs. concentration of NPs and supernatants (control), from all measurements, with their standard deviations. We used conventional statistical analysis methods (t-test) to determine the significance of the different observations of normally developed, deformed and dead zebrafish between different concentrations (0 - 1.2 nM) of Au NPs in Figure 11A. We found significant differences of normally developed, deformed and dead zebrafish observed at 0.20 nM Au NP concentration from those observed in the absence of NPs (0 nM, control experiments) with a confidence level of 90% (or $P = 0.10$). We observed a significant difference in the number of deformed zebrafish (but not normally developed or dead zebrafish) at 0.20 nM from those at other concentrations (0.40, 0.60, 0.80, 1.0, or 1.2 nM) with a confidence level of 90% (or $P = 0.10$), but no significant differences among other concentrations.

We report all unique observations, especially rare deformed zebrafish, in Figure 12C. We have never observed the deformed zebrafish in the absence of Au NPs (control experiments). All rare events presented in this study are within
the confidence level of 95% (or \( P = 0.05 \)) for four sets of measurements (each with 15 – 20 embryos) at each NP concentration, analyzed using conventional statistical methods (e.g., t-test or Q-test). Note that study of embryos at the single embryo level allows us to depict the rare event of interest, which otherwise would be buried under ensemble measurements, emphasizing the importance of the study of a bulk amount of embryos at the single-embryo level.

We investigated over 100 NPs for each measurement of single NPs to gain sufficient data for statistical analysis and for determining the size distribution and color distribution that represents the bulk NP solution at the single NP resolution. We repeat all measurements, including characterization of photostability and LSPR spectra of single NPs, control experiments, and analysis of concentrations of NP solutions, at least three times, and present representative and average data of all measurements with standard deviations. All measurements are very reproducible, well beyond the confidence level of 95% (or \( P = 0.05 \)). Thus, we do not need to reject any data.
CHAPTER III

DEVELOPMENT OF ULTRASENSITIVE IN VIVO ASSAYS FOR STUDY OF BIOCOMPATIBILITY AND TOXICITY OF 86 NM GOLD NANOPARTICLES

INTRODUCTION

Noble metal NPs (e.g., Au and Ag NPs) show distinctive plasmonic optical properties, superior photostability, and high quantum yields (QYs) of Rayleigh scattering.\textsuperscript{1,2,16,19} The QY here refers to the ratio of scattering intensity (number of scattering photons) of a single NP to intensity of its incident light. Plasmonic optical properties of the single NPs highly depend upon their sizes, shapes, dielectric constants, and surrounding environments.\textsuperscript{12-19} We have used localized surface plasmon resonance (LSPR) spectra of single Au and Ag NPs to determine their sizes, shapes, surface properties, and surrounding environments at single NP resolution.\textsuperscript{21, 22, 52-54, 56-60, 79-85} Unlike fluorescence molecules or quantum dots (QDs), single plasmonic NPs resist photobleaching and photoblinking, and can be imaged and characterized in single live cells and embryos for any desired period of time.\textsuperscript{20-22, 52-54, 56-60, 79-85} Thus, they are most suitable for the study of dynamic events of interest \textit{in vivo} in real-time.

To serve as effective imaging probes and sensors for study of biological functions in living organisms, it is essential that these plasmonic NP probes are biocompatible and can penetrate living organisms on their own (non-invasively). Despite extensive study of Au NPs and their potential applications in biology and medicine,\textsuperscript{2, 8} their effects on living organisms and their dependence upon physicochemical properties of the NPs still remain largely unknown.\textsuperscript{1,28,49,50}

Currently, \textit{in vitro} studies account for the majority of nanotoxicity research.\textsuperscript{8,41-51} However, the results from \textit{in vitro} studies can be misleading. For example, \textit{in vitro} assays may not capture critical insights into the effects of NPs upon intercellular functions and their related biological functions \textit{in vivo}. Certain nanomaterials may interfere with the readout of common cytotoxicity assays due
to their interactions with assay reagents, and their overlapping absorption, scattering or emission properties. A wide range of NP concentrations, exposure times, cell lines, and cell culture conditions have been used among various in vitro studies, which make direct comparison among the studies extremely difficult.8, 41-51 Furthermore, many studies used unpurified and unstable (aggregated) NPs, or the NPs coated with different surface functional groups, and these studies did not consider the potential toxic effects of surface functional groups, aggregations of NPs, and other contaminant chemicals (impurities) on the cells. Majority of studies did not characterize the physical properties (e.g., sizes, doses) of NPs throughout the entire duration of experiments in situ in real-time. Thus, various studies created a wide range of inconclusive, contradictory or misleading results.

To overcome the limitations of current nanotoxicity studies, we have developed ultrasensitive in vivo assays (cleavage stage zebrafish embryos) to characterize biocompatibility and toxicity of nanomaterials.53, 57-60, 85 To depict the dependence of NP toxicity on their physicochemical properties, and to rationally design biocompatible NP imaging probes, we have synthesized a mini-library of Au and Ag NPs with various sizes and surface functional groups, purified them, determined their stability, and developed DFOMS to characterize them throughout their incubation with living organisms in situ in real-time at single NP resolution.20-22, 52-54, 58-60, 79-85

In our previous studies, we used cleavage stage zebrafish embryos for study of the transport, biocompatibility and toxicity of purified Ag NPs with three distinctive sizes (11.6 ± 3.5, 41.6 ± 9.1, and 95.4 ± 16.0 nm) and the smaller Au NP (11.6 ± 0.9 nm).53, 58, 60, 85 In this study, we use zebrafish embryos to characterize the transport, biocompatibility and toxicity of the larger Au NPs (86.2 ± 10.8 nm), aiming to determine the dependence of biocompatibility and toxicity of the NPs upon their sizes and chemical compositions for rational design of biocompatible NP probes.
RESULTS AND DISCUSSION

Synthesis and characterization of purified and stable Au NPs

We synthesized the Au NPs (86.2 ± 10.8 nm) by reducing HAuCl₄ with sodium citrate. The colloidal Au NPs were thoroughly washed three times using centrifugation to remove any trace chemicals from the synthesis to prepare highly purified Au NPs. We suspended purified Au NPs in egg water (1.0 mM NaCl in DI water, embryonic medium) and characterized their sizes, plasmonic optical properties and concentration in egg water over the entire embryonic development (120 h) using high-resolution TEM (HR-TEM), dynamic light scattering (DLS), DFOMS, and UV-vis absorption spectroscopy, respectively.

The TEM images and histograms of size distributions of single Au NPs (Fig. 14A - B) show the ellipse shaped NPs with average sizes of 85.4 ± 11.6 and 86.2 ± 10.8 nm of the Au NPs dispersed in egg water at 0 and 120 h, respectively. The results show that the sizes of the NPs remain essentially unchanged over their 120 h incubation with egg water. The average sizes of NPs are calculated by averaging the length and width of each NP.

We also characterize the sizes of Au NPs in egg water over 120 h using DLS. The results (Fig. 14C) show their average sizes (diameters) of 86.3 ± 9.0 and 87.9 ± 9.4 nm at 0 and 120 h, indicating that the sizes of the NPs remain essentially unchanged and the NPs are very stable (non-aggregated) over their 120 h incubation with egg water. The sizes of hydrated NPs measured using DLS (Fig. 14C) are slightly larger than those measured by TEM (Fig. 14A - B). Nonetheless, the sizes of the ellipse shaped NPs determined by DLS are calculated using equations for spherical NPs. Thus, one cannot directly compare the hydrated ellipse shaped NPs measured by DLS, with their sizes determined by TEM and calculated as the average of their lengths and widths. Notably, the sizes of NPs determined by both DLS and TEM are quite close; indicating that the approximation of ellipse shaped NPs as spherical NPs for ensemble DLS
measurement is acceptable. In this study, we select the sizes of the NPs determined by TEM to describe the sizes of the NPs.

We also study the concentrations and stability of the Au NPs in egg water over 120 h by measuring their UV-vis absorption spectroscopy (ensemble measurements). The absorption spectra of the NPs in egg water (20 pM) over 120 h show that their base-line subtracted peak absorbance of 0.53 at 550 nm, $\lambda_{\text{max}}$ (FWHM = 102 ± 4 nm), remain unchanged (Fig. 14D), which further demonstrates that the Au NPs are stable in egg water over 120 h. If the NPs were aggregated (unstable), the absorbance of their UV-vis absorption spectra would decrease and their peak wavelength would be red shifted. But that was not what we observed. Thus, the NPs remain stable (non-aggregated) in egg water over 120 h.

We further characterize plasmonic optical properties of single Au NPs and their stability in egg water over 120 h at single NP resolution using DFOMS. Dark-field optical image of single Au NPs and histograms of distributions of plasmonic colors (LSPR spectra of single NPs) show that majority of single NPs exhibit plasmonic green with some being orange and red, which remains essentially unchanged over their 120 h incubation with egg water (Fig. 15A - C). We determine the number of single NPs in egg water (concentrations at single NP resolution) over 120 h using DFOMS. Unlike UV-vis spectroscopy (ensemble measurement), DFOMS enables us to image the number of single NPs in a given detection volume (one full-frame CCD image) to determine the concentration of the NPs at single NP resolution. The number of single NPs (20 pM Au NPs) in 60 images acquired by DFOMS at each give incubation time is (257 ± 2) NPs per image (Fig. 15D), demonstrating that the NPs in egg water are very stable (non-aggregated). If the NPs were aggregated (unstable), their number in solution would have decreased over time. But that was not what we observed. Thus, the NPs remain stable (non-aggregated) in egg water over 120 h. Representative LSPR spectra of single Au NPs in egg water (Fig. 15E) show $\lambda_{\text{max}}$ (FWHM), at 554 (105), 619 (97), and 683 (82) nm, which remain essentially unchanged.
Figure 14. Characterization of sizes and stability (non-aggregation) of the Au NPs suspended in egg water.

(a) HR-TEM images of the Au NPs that had been suspended in egg water for: (A) 0 and (B) 120 h, showing single ellipse shaped NPs. (b) Histograms of the size distributions of single Au NPs measured by HR-TEM show average sizes of NPs: (A) 85.4 ± 11.6 and (B) 86.2 ± 10.8 nm. (C) Histograms of size distributions of the NPs dispersed in egg water and measured by DLS show that their average sizes are (a) 86.3 ± 9.0 and (b) 87.9 ± 9.4 nm for 0 and 120 h, respectively. (D) UV-vis absorption spectra of the Au NPs in (C) at: (a) 0 and (b) 120 h, showing that the λ_max (FWHM) at 550 (102 ± 4) nm remains unchanged over time. Scale bars = 100 nm.
We characterize the stability of a dilution series of the purified Au NPs using the above approaches, to determine their "solubility" (a maximum concentration) that the Au NPs are stable in egg water over 120 h. We then use the concentrations of the stable and "soluble" (non-aggregated) Au NPs in egg water to incubate with cleavage stage embryos, follow embryonic development over 120 h, and study the effects of NPs on embryonic development. Unlike other studies, we use DFOMS to characterize the stability (LSPR spectra and number) of Au NPs in egg water at single NP resolution in situ in real-time, which is much more sensitive and accurate than any conventional ensemble measurements (e.g., UV-vis absorption spectroscopy).

We correlate the distribution of sizes of single Au NPs measured by TEM with the distribution of plasmonic colors (LSPR spectra) of single Au NPs in egg water determined by DFOMS. At 120 h, the results from TEM measurements show that the sample includes 63% of 60 - 88 nm, 28% of 89 - 100 nm, and 9% of 101 - 118 nm NPs (Fig. 14B). Dark-field optical image of single Au NPs (Fig. 15C: b) shows that majority of single NPs exhibit plasmonic green (65 ± 4%) with some being orange (28 ± 1%) and red (7 ± 3%). Note that TEM samples are prepared from the same Au NP solutions as those measured by DFOMS. Thus, for this Au NP solution, the NPs with sizes of 60 - 88, 89 - 100, and 101 - 118 nm show plasmonic green, orange, and red, respectively.

Taken together, the results in Figures 14 and 15 show that the Au NPs (20 pM) are very stable (non-aggregated) in egg water over the entire duration of embryonic development (120 h), which enables us to study their size- and dose-dependent transport into/in embryos and their effects on embryonic development in vivo in real-time.
Figure 15. Characterization of the plasmonic optical properties and stability (non-aggregation) of single Au NPs suspended in egg water.

(A, B) Dark-field optical images of the Au NPs that had been suspended in egg water for: (A) 0 and (B) 120 h, showing single plasmonic green, orange and red NPs. Scale bar represents 2 µm, which shows the distance among individual NPs, but not the sizes of NPs, because they were imaged under optical diffraction limit. (C) Histograms of color distributions of the single Au NPs in (A, B) measured by DFOMS show: (a) 70 ± 4% of plasmonic green, 23 ± 5% of orange and 7 ± 2% of red NPs; and (b) 72 ± 5% of plasmonic green, 22 ± 6% of orange and 5 ± 1% of red NPs. (D) The number of single Au NPs imaged by DFOMS is 264 ± 10, 261 ± 9, 250 ± 6, 249 ± 8, 257 ± 13, and 261 ± 7 NPs per image at 0, 24, 48, 72, 96, and 120 h, respectively. (E) LSPR spectra of single plasmonic green, orange and red Au NPs show λ_{max} (FWHM) at: (a) 554 (105); (b) 619 (97); and (c) 683 (82) nm.
Real-time imaging of transport of the single Au NPs into/in embryos

We incubated the purified and stable Au NPs (20 pM) with the cleavage stage zebrafish embryos and imaged the diffusion and transport of single NPs into the embryos and inside the embryos in vivo in real-time, aiming to determine whether such larger Au NPs (60 - 118 nm) can transport into living embryos and their transport mechanism. The snap-shots of sequential optical images (Fig. 16) show arrays of chorionic pores on chorionic layers (CLs) and their diameters (0.5 - 0.7 μm) and that single Au NPs diffuse through the chorionic pore canals into the chorionic space (CS), and through the CS into the inner mass of embryos (IME). We characterized the LSPR spectra of single Au NPs, as those shown in Figure 15E, to distinguish single Au NPs over embryonic tissues and debris, which appear white under dark-field illumination.

Representative diffusion trajectories of single Au NPs (Fig. 17a), each with an identical green color (size), show that single Au NPs are diffusing (A) into the embryo via chorionic pores; (B) in the CS; and (C) into the IME from the CS. The diffusion modes of these single Au NPs in various locations of the developing embryos are further characterized by plotting real-time square-displacement (RTSD) of single Au NPs vs. diffusion time (Fig. 17b). Note that viscosities inside a developing embryo vary widely upon local embryonic environments, which also alter rapidly with time as the embryo develops. Therefore, we use RTSD (diffusion distance at each time interval) instead of MSD (average distance over time), to determine the diffusion modes and diffusion coefficients of single NPs, as they diffuse into and through embryos.

Using diffusion theories,73-75 we found restricted diffusion for the NPs that diffused from egg water through the chorionic pores into the CS (Fig. 17A: b). Several steps in the plot suggest that the NPs become trapped (2 - 8 s) within the pores from time to time, possibly due to their interactions with the pores. Nonetheless, the linear plot shows stationary random Brownian diffusion as a diffusion mode for the NPs entering into the embryos. Thus, passive diffusion,
Figure 16. Real-time imaging of transport of single Au NPs into cleavage stage zebrafish embryos using DFOMS.

(A) Optical image of the cleavage stage embryo in egg water shows its CL, CS and the interface of the CS and the IME, as highlighted by (B – D), respectively.

(B – D) Snapshots of sequential dark-field optical images of the zoomed-in areas squared in (A) show the diffusion of single Au NPs (circled) from the egg water (outside chorion, OC) into the CL, in the CS, and at the interface between the CS and IME, respectively. The temporal resolution of real-time imaging is 100 ms. Scale bars = 200 μm in (A) and 5 μm in (B – D).
but not active transport, is responsible for the NPs to enter the embryos. The diffusion coefficients (D) are determined by dividing the slope of the plot of RTSD versus time by 4 using two-dimension random walk theory (RTSD = 4DΔt). The diffusion coefficients vary drastically as the NPs diffuse from the egg water through the chorionic pores and into the CS with an average of \(1.7 \pm 1.5 \times 10^{-9}\) cm\(^2\) s\(^{-1}\). Their larger standard deviation reflects highly heterogeneous embryonic environments.

The plots of RTSD vs. time for single Au NPs that diffuse in the CS and from CS into the IME also exhibit linearity with larger slopes and fewer steps (Figs. 17B - C), showing simple random Brownian diffusion and suggesting that transport barrier at the CL is much higher than the interface of CS and IME. Their average diffusion coefficients in (B) and (C) are \(6.3 \pm 2.7 \times 10^{-9}\) and \(5.1 \pm 2.1 \times 10^{-9}\) cm\(^2\) s\(^{-1}\), respectively. Notably, the slopes of the plots in (C) change over time much more notably and they are smaller than those in (B), suggesting higher viscosity gradients at the interface of CS into IME, than those in CS.

We use the same approaches to study the diffusion of plasmonic green Au NPs in egg water, which shows random Brownian diffusion with diffusion coefficients of \(1.6 \pm 0.2 \times 10^{-8}\) cm\(^2\) s\(^{-1}\). Note that diffusion coefficients of single NPs are inversely proportional to viscosities of medium, as described by Stokes-Einstein equation, \(D = kT/(6\pi\eta a)\), for a spherical NP, where \(k\) is Boltzmann constant; \(T\) is temperature; \(a\) is radii of single NPs; and \(\eta\) is viscosity of medium where NPs diffuse in.\(^{76}\) Thus, the results show that embryonic environments are orders of magnitude more viscous than egg water. These results agree well with those we reported previously using similar sized Ag NPs,\(^{60}\) showing highly heterogeneous embryonic environments with larger viscosity gradients.

Taken together, the results in Figure 17 show that the same sized Au NPs diffuse 14.96, 58.36 and 42.44 μm\(^2\) within the same duration of measurement (18.73 s) as the NPs diffuse across CL, in the CS and into IMS, suggesting the highest restriction at the CL and the lowest viscosity in CS.
Figure 17. Real-time study of the diffusion modes of single Au NPs in cleavage stage embryos.

(a) Diffusion trajectories and (b) plots of RTSD vs. time of single plasmonic green Au NPs that diffuse: (A) from the egg water into the CS; (B) in the CS; and (C) at the interface between the CS and IME indicate restricted diffusion in (A) and random Brownian motion in (B, C) with diffusion coefficients of $1.7 \pm 1.5 \times 10^{-9}$, $6.3 \pm 2.7 \times 10^{-9}$ and $5.1 \pm 2.1 \times 10^{-9}$ cm$^2$ s$^{-1}$, respectively. Each pixel is 67 nm.
Study of dose-dependent biocompatibility of Au NPs

To study the dose-dependent biocompatibility and toxicity of the Au NPs, we incubated the cleavage stage embryos (0.75 - 2.25 hpf) with various concentrations (0 - 20 pM) of the purified and stable Au NPs in egg water, and the supernatant collected from last washing of the NPs over 120 h. We imaged the developing embryos every 24 h until they fully developed at 120 hpf. We characterized normally developed zebrafish (Fig. 18A), deformed zebrafish (Fig. 18B), and dead embryos (Fig. 18C) using their morphologies and phenotypes. The number of embryos that developed to normal and deformed zebrafish or became dead was plotted against molar concentrations of the NPs and supernatants that were incubated with the embryos (Fig. 18D). The study of embryonic development in the supernatant serves as control experiments to determine effects of potential residual chemical contaminants on embryonic development, which ensure that the death and deformities of embryos exposed to the NPs are not caused by any potential residual chemicals from the NP synthesis.

This experimental design enables the NPs to passively diffuse into embryos without external intervention (non-invasively) and to study their effects on embryonic development in vivo in real-time. These approaches also mimic the potential transport and effects of the NPs on aquatic and eco-living organisms should the NPs release to the environments. We select the cleavage stage embryos as in vivo assays to study the effects of the NPs on embryonic development because the embryonic cells at this given stage synchronously divide and undergo molecular changes that ultimately affect the fates of developing embryos.

The results show that embryonic development insignificantly depends upon the NP concentration (dose) (Figs. 18D - E). As the NP concentration increases from 0 to 20 pM, the number of embryos that develop to normal zebrafish remain essentially unchanged at 96 ± 4%, and the number of embryos
that develop to deformed zebrafish or become dead also remain the same at 1 ± 1% and 3 ± 3%, respectively. The statistical analysis of the data using ANOVA with a confidence level of 95% (P = 0.05) reveals insignificant differences of death and deformed embryos as they are exposed to various concentrations of the Au NPs (0 - 20 pM), egg water or supernatant. Notably, the percentages of embryos that are incubated with the supernatant over 120 h and develop to normal zebrafish or become dead are the same as those in egg water alone (blank control) (Fig. 18E) and none of embryos develop to abnormal zebrafish, showing that the death and deformities observed in Figure 18D are not caused by any potential residual chemicals from the NP synthesis.

The chronic treatment of embryos with the Au NPs over 120 h results in only two abnormally developed zebrafish (Fig. 18B), which are rare events (the outliers) of the measurements and can only be detected at single embryo resolution. Both of them display more than one type of deformation, and the types of deformation are independent upon the NP concentration. For example, the embryo that has been incubated with the lower NP concentration (2.4 pM) for 120 h, develops to the deformed zebrafish with all types of deformities (finfold abnormality, tail and spinal cord flexure, cardiac malformation, yolk sac edema and eye abnormality). In contrast, the embryo that has been incubated with the higher NP concentration (9.9 pM) for 120 h, develops to the deformed zebrafish with finfold abnormality, and tail and spinal cord flexure. These observations suggest that some embryos may be more vulnerable (sensitive) to the effect of the Au NPs on their development, while other embryos may be more tolerate (resistant) to the effect of the Au NPs. Notably, each embryo is quite distinctive, and it is common that a small percentage of the embryos become dead during the development under the same incubation conditions (in egg water). Random diffusion of single Au NPs into the various compartments of the embryos, especially at such low concentrations, may also cause the rare and stochastic toxic effects on embryonic development, as we reported previously.\textsuperscript{85}
Figure 18. *In vivo* study of the effect of Au NPs on embryonic development. (A) Optical images of a normally developed zebrafish show normal development of the cardiac region, yolk sac, head and eyes. (B) Optical images of deformed zebrafish displaying several deformities, including (a) finfold abnormality, tail/spinal cord flexure, cardiac malformation, yolk sac edema and eye abnormality; and (b) finfold abnormality and tail/spinal cord flexure; and (C) dead embryo. (D) Histograms of the percentages of the embryos that developed to normal and deformed zebrafish or became dead vs. NP concentration. (E) Histograms of the percentages of embryos that developed to normal zebrafish or became dead either in egg water alone or in supernatant. Scale bars = 250 µm in (A), 500 µm in (B) and 200 µm in (C).
Quantitative imaging of single Au NPs in tissues of normal and deformed zebrafish

To determine why some embryos developed normally while others became deformed or dead, we imaged single Au NPs embedded in the tissues of normally developed and deformed zebrafish and quantitatively characterized distributions of single Au NPs in the tissues using DFOMS. We incubated the cleavage stage embryos with the Au NPs (0 - 20 pM) continuously (chronically) for 120 h until they developed to normal or deformed zebrafish. We then rinsed and fixed the normally developed and deformed zebrafish and prepared the ultrathin sections (1 - 4 μm thickness) of their tissue samples. Individual Au NPs embedded in various tissues of normal and deformed zebrafish were characterized using DFOMS.

We observed the green, orange and red Au NPs embedded in different locations of normally developed zebrafish (Fig. 19A - C), including (a) retina, (b) gill slits and (c) tail muscles. The LSPR spectra of single Au NPs embedded in the tissues (Fig. 19B) show characteristic plasmonic \( \lambda_{\text{max}} \) at 555 nm (green) and 697 nm (red), enabling us to distinguish the Au NPs from the tissues. Single Au NPs were also observed in different locations of a deformed zebrafish (Fig. 19D - F), including (a) retina, (b) yolk sac and (c) tail muscles. LSPR spectra of single Au NPs embedded in the tissues (Fig. 19E) show distinctive \( \lambda_{\text{max}} \) at 579 nm (green) and 738 nm (red). These results suggest that the NPs diffuse into the developing embryo (Figs. 16 and 17) and remain inside the embryos throughout their developmental stages (120 h). The red shift of the LSPR spectra of single Au NPs could be attributed to the embryonic tissues, which decrease the surface refractive index of the NPs and offer low dielectric constants than the egg water. Nonetheless, the potential aggregation of a very few of single Au NPs embedded in the tissues during their incubation with embryos over 120 h could also lead to the larger NPs and red shift of their LSPR spectra.
Figure 19. Quantitative imaging of single Au NPs embedded in a normally developed and deformed zebrafish using DFOMS.

(A) Optical image of an ultrathin longitudinal section of a fixed zebrafish. The rectangles highlight its (a) retina (eye), (b) gill slits and (c) tail muscle. (B) LSPR spectra of single Au NPs embedded in the tissues show distinctive λ_max (FWHM) at (a) 555 (157) and (b) 697 (174) nm. (C) Zoomed-in optical images of those outlined in (A) show single Au NPs embedded in the tissues, as circled. (D) Optical image of an ultrathin longitudinal section of a fixed deformed zebrafish. The rectangles outline its (a) retina (eye), (b) yolk sac and (c) tail muscle. (E) LSPR spectra of single Au NPs embedded in the tissues show distinctive λ_max (FWHM) at (a) 579 (186) and (b) 738 (194) nm. (F) Zoom-in optical images of those outlined in (D), show individual Au NPs embedded in the tissues, as circled. Scale bars = 200 µm in (A, D) and 10 µm in (C, F).
We quantitatively analyzed the number and sizes of individual Au NPs embedded in the tissues using their distinctive plasmonic optical properties (colors, LSPR spectra), and size-dependent LSPR spectra. Plots of number of given plasmonic colors (sizes) of single NPs in normal and deformed zebrafish indicate that more NPs are embedded in the eye and tail tissues of the deformed zebrafish than those of the normally developed zebrafish (Fig. 20). The percentages of plasmonic green and red NPs embedded in the tissues are similar to those observed in solution (Fig. 15), which suggests that the NPs remain stable (non-aggregated) throughout their incubation with embryos in egg water over 120 h. The larger number of Au NPs accumulated inside the embryos throughout their development may lead to their deformation and dead. Nonetheless, individual embryos might have various degrees of tolerance to the effects of the Au NPs. Embryos with the high degree of tolerance could survive and develop to normal zebrafish. In contrast, those with the low degree of tolerance could develop to deformed zebrafish or become dead. Such individuality phenomena could not be detected if ensemble measurements were utilized. Therefore, this study further underscores the importance of study of effects of the NPs on embryonic development at single NP and single embryo resolutions.

Comparing with our previous studies: size and chemical dependent toxicity of Au and Ag NPs

To compare with our previous study of the effect of smaller Au NPs (11.6 ± 0.9 nm; 0 - 1.2 nM) on the embryonic development, we plot the percentages of embryos developed to normal or deformed zebrafish vs. surface area of Au NPs in a given solution volume (Fig. 21). The surface areas of two different sized Au NPs are used to enable a full scale comparison. The results show dose-independent effects of both smaller and larger Au NPs upon embryonic development. Interestingly, the results clearly reveal size-dependent biocompatibility and toxicity of Au NPs upon the embryonic development. The larger Au NPs cause fewer embryos to become dead than the smaller Au NPs,
Figure 20. Quantitative analysis of the number and sizes of single Au NPs embedded in the tissues of normal and deformed zebrafish.

Histograms of the number and sizes (colors) of single NPs embedded in (A) retina (eye) and (B) tail muscle of (a) normal and (b) deformed zebrafish.
suggesting that the larger Au NPs (86.2 ± 10.8 nm) are more biocompatible than smaller Au NPs (11.6 ± 0.9 nm) at the same given doses.

In contrast, our previous studies of effects of purified and stable Ag NPs (11.6 ± 3.5; 41.6 ± 9.1; and 95.4 ± 16.0 nm with similar doses to the Au NPs) on the embryonic development show much higher toxicity of Ag NPs than the Au NPs.\textsuperscript{53, 58, 60} The toxicity of Ag NPs highly depends upon their doses and sizes.\textsuperscript{53, 58, 60} For the same sized Ag NPs, higher doses of Ag NPs cause more embryos to become dead or to develop to more severely deformed zebrafish. For the same doses of Ag NPs, the larger Ag NPs cause more embryos to become dead and to develop to more severely deformed zebrafish than smaller ones.

By comparing with these previous studies of smaller Au NPs and various sized Ag NPs,\textsuperscript{53, 58, 60, 85} we found the size-dependent and chemical-dependent biocompatibility and toxicity of the Au and Ag NPs. Notably, our previous studies neither predict nor conclude the effects of larger Au NPs (86.2 ± 10.6 nm) on the embryonic development. It is entirely unknown whether such larger Au NPs can be stable in egg water, whether they can enter the embryos and whether they are more biocompatible or toxic than the smaller Au or Ag NPs. Thus, all findings from this study are distinctive and cannot be extrapolated from any previous studies. In this study, we use the exactly same approaches (e.g., same stage of embryos, same exposure conditions, and same characterization methods), as those in the previous studies, except different sized Au NPs. Such approaches are essential for us to determine the biocompatibility and toxicity of the NPs upon their sizes and chemical properties.

Molecular mechanisms of size and chemical dependent effects of Au and Ag NPs upon embryonic development are currently under investigation. It is most likely that physicochemical properties of the NPs, including their surface properties, sizes, shapes, doses, and chemical compositions, create combined and complex effects on embryonic development, and each physicochemical property could create distinctive effects on embryonic development at a given
Figure 21. Study of size-dependent biocompatibility and toxicity of Au NPs on embryonic development.

Percentages of embryos that were incubated with given concentrations of NPs in egg water for 120 h (A) developed to normal zebrafish or (B) became dead, vs. the surface area of NPs per a given volume (dose) for: (a: filled triangles) 86.2 ± 10.8 nm; and (b: open triangles) 11.6 ± 0.9 nm Au NPs.85
condition. Such effects may not be proportional to the sizes of the NPs, and may well depend upon the surface properties and chemical reactivity of the NPs. These effects could be dependent upon living organisms as well. Thus, it is important to study the effects of each physicochemical property of the NPs on a given living organism one at a time, in order to understand their physicochemical dependent nanotoxicity and rationally design biocompatible nanomaterials. It is worth noting that the conclusions reported in this study are specific for the sizes and shapes of the NPs used in this study, and cannot be generally applied to other NPs or extrapolated to other sizes of Au NPs. In other words, size and chemical dependent biocompatibility and toxicity of Au and Ag NPs may not be linear, and further studies would be needed. Our studies suggest that it is very likely that each size of the Au or Ag NPs possess its own properties.

Taken together, these important findings offer interesting new insights into the size-dependent biocompatibility of Au NPs, and chemical-dependent nanotoxicity of Au and Ag NPs at single NP resolution. Such studies are essential for rational design of biocompatible nanomaterials for a wide range of applications, including in vivo imaging, drug delivery and implant devices. For example, the smaller NPs would be easier to penetrate into the sub-compartments of living organisms to enable in vivo imaging and sensing for effective diagnosis, while the larger NPs could carry larger pay-load of therapeutic agents to enable effective drug delivery and achieve effective therapy. We have demonstrated that DFOMS-MSIS is a powerful imaging tool for real-time in vivo imaging of transport and effects of single Au NPs, and for quantitative analysis of individual Au NPs embedded in the tissues. This study further demonstrates that LSPR spectra of single Au NPs can be used to study size-dependent biocompatibility and nanotoxicity. One can now use the similar approaches, such as single NP fluorescence or Raman spectroscopy, to study transport and toxicity of nanomaterials with given optical and spectral properties.
SUMMARY

In summary, we have synthesized and characterized purified and stable (non-aggregated) Au NPs (86.2 ± 10.6 nm) and developed DFOMS-MSIS to study their transport into/in embryos and their effects on embryonic development in vivo in real-time at single NP resolution. We found that single Au NPs passively diffused into the chorionic space of the embryos via their chorionic pore canals and continued into the inner mass of embryos. The diffusion coefficients of single Au NPs range from $1.7 \times 10^{-9}$ to $6.3 \times 10^{-9}$ cm$^2$ s$^{-1}$, as single Au NPs passively diffuse through different locations of the developing embryos, suggesting diverse viscosity gradients and transport barriers within the embryos. The majority of embryos (96%) that are chronically incubated with the Au NPs (0 - 20 pM) over 120 h, develop to normal zebrafish, while insignificant amounts of embryos either become dead (3%) or develop to deformed zebrafish (1%), which are independent upon the dose of Au NPs. The Au NPs are embedded in various tissues of normal and deformed zebrafish, and more Au NPs are observed in the deformed ones. The results show that the Au NPs diffuse into the embryos and stay inside the embryos throughout their development (120 h), and random walk of single Au NPs may be attributed to their stochastic toxic effects on embryonic development. By comparing with our previous study of the smaller Au NPs (11.6 ± 0.9 nm) and various sized Ag NPs (11.6 ± 3.5; 41.6 ± 9.1; and 95.4 ± 16.0 nm), we found the size-dependent biocompatibility of Au NPs and chemical-dependent biocompatibility and nanotoxicity of Au and Ag NPs. At the same given doses, the larger Au NPs (86.2 ± 10.6 nm) are slightly more biocompatible with embryonic development than the smaller Au NPs (11.6 ± 0.9 nm), while the similar sized Ag NPs are much more toxic than the Au NPs. Note that we observe similar diffusion coefficients of the same sizes of Ag and Au NPs into/in embryos. Thus, the size-dependent biocompatibility of the Au NPs is very unlikely attributed to their size-dependent accumulation inside the embryos. Taken together, this study offers new tools and new insights for the study of transport and toxicity of the Au NPs in vivo in real-time at single NP resolution,
which are essential to the rational design of biocompatible NPs for a wide variety of biomedical applications, including in vivo imaging, drug delivery and implant devices.

METHODS

Reagents and supplies

All chemicals were purchased from Sigma and used as received, and the DI water (18 MΩ, Barnstead) was used to prepare solutions and rinse glassware.

Synthesis and characterization of stable and purified Au NPs (86.2 ± 10.8 nm)

We synthesized the Au NPs (86.2 ± 10.8 nm) by reducing a freshly prepared HAuCl₄ solution (520 mL, 0.01% w/v, in DI water) with a freshly prepared sodium citrate solution (2.2 mL, 1.1% w/v).38, 71, 87 The HAuCl₄ in a three-necked round bottom flask was heated to the boiling temperature (100°C) under refluxing and stirring, and the sodium citrate solution was rapidly added into the flask. After the color of the solution changed from yellow to colorless, to dark purple and finally to dark red, we continued stirring and refluxing the solution for another 15 min. We then stopped heating the solution until it was cooled to room temperature. The NPs were immediately filtered using 0.22 μm filters, and washed three times DI water using centrifugation to remove the by-products and any excess chemicals from NP synthesis.

We first spun down the NPs using centrifugation (1000 RPM, DYNAC II, Clay Adams, 1 h). We imaged the supernatant using DFOMS to ensure that there were not any NPs in the supernatant (all of NPs were spun down). If we found that any single NPs were present in the supernatant, we would increase the speed or time of centrifugation or use ultracentrifugation system (Beckman L90) to remove them from the supernatant. We then removed the supernatant, and resuspended the NPs (pellet) in DI water via vortex. We repeated such a
washing step three times. The supernatant removed from the last washing step was collected and used to incubate with embryos over 120 h, which served as a control experiment to determine the effects of any possible trace chemicals involved in NP synthesis on embryonic development.

We re-suspended the pellet of the purified Au NPs in egg water and characterized their sizes, concentrations and optical properties over 120 h to determine their stability using HR-TEM (JEOL, JEM-2100 F), DLS (Nicomp 380ZLS particle sizing system), UV-vis spectroscopy (Hitachi U2010), and DFOMS (Figs. 14 - 15), respectively. The TEM samples were prepared by adding one drop of Au NP suspension (5 μL) onto each TEM grid, which was dried rapidly. We repeated such a process 2 - 3 times to ensure that each TEM grid was covered with sufficient Au NPs for effective TEM imaging (Figs. 14A - B). For DLS measurements (Fig. 14C), the average sizes of the NPs (20 pM, 700 μL of Au NPs suspended in egg water in a borosilicate tube) were measured seven times at each given time over 120 h at room temperature using DLS (ensemble measurement). The purified and stable Au NPs were used for probing of their diffusion into embryos and for study of their effects on embryonic development over 120 h.

For DFOMS measurements (Fig. 15), we sampled the Au NPs suspended in egg water into a home-made microchamber, and directly acquired LSPR spectra (colors) and number of single Au NPs in the microchamber in real-time using DFOMS, as we described previously.\textsuperscript{20-22, 52-54, 56-60, 79-85} Histograms of plasmonic spectra (colors) of single Au NPs in egg water at each given time (Fig. 15C) were correlated with the sizes of single Au NPs measured by TEM, because TEM samples were prepared from the same Au NP samples in egg water at the same give incubation time. We have fully described the designs and applications of our DFOMS for real-time imaging and spectroscopic characterization of single NPs in solutions, single live cells and embryos, and for single molecule detection.\textsuperscript{20-22, 52-54, 56-60, 79-85} In this study, dark-field microscope coupled with CCD camera (EMCCD or Coolsnap HQ2) and a spectrograph
(SpectraPro-150, Roper Scientific), or Multispectral Imaging System (MSIS, N-MSI-VIS-FLEX, CRi, Hopkiton, MA) are used to image and characterize LSPR spectra of single Ag NPs. The MSIS is an integrated system of a CCD camera (SonyICX 285) and liquid-crystal-tunable-filter (LCTF). DFOMS-MSIS can simultaneously acquire dark-field plasmonic optical images and spectra of massive amount of individual NPs with spectral resolution of 1 nm, and enable high-throughput spectral analysis and characterization of single NPs. The dark-field microscope is equipped with a dark-field condenser (oil, 1.43 - 1.20), a microscope illuminator (Halogen lamp, 100 W), and a 100x objective (Plan fluor 100x, N.A. 0.5-1.3, oil), as we described previously.

**Breeding and monitoring of development of zebrafish embryos**

We housed wild-type adult zebrafish (Aquatic Ecosystems) in a stand-alone system (Aquatic Habitats), maintained and bred zebrafish as described previously. Briefly, we placed two pairs of mature zebrafish into a clean 10 gallon breeding tank, and used a light (14 h) - dark (10 h) cycle to trigger breeding and fertilization of embryos. We collected the embryos at 2 hpf, transferred them into a petri dish, and well rinsed them with egg water to remove the surrounding debris. The washed embryos were then used for real-time imaging of the diffusion and transport of single Au NPs into/in embryos and for quantitative study of their effects on embryonic development. All experiments involving embryos and zebrafish were conducted in compliance with the IACUC guidelines (protocol #11-030).

**In vivo real-time imaging of diffusion and transport of single Au NPs into/in embryos**

We incubated the cleavage stage embryos with the purified Au NPs (20 pM) in home-made imaging microchambers, and imaged the diffusion of single Au NPs into embryos in real time using our DFOMS (Figs. 16 - 17). Distinctive plasmonic optical properties (colors) of single Au NPs enable them to be distinguished from embryonic debris and zebrafish tissues, which appear white.
under dark-field illumination. LSPR spectra of single Au NPs were acquired by DFOMS-MSIS (Fig. 15E), as we described previously. 53, 57-60, 79, 82, 84, 85, 89, 90

**Study of dose-dependent biocompatibility and toxicity of Au NPs (86.2 ± 10.8 nm)**

We incubated the cleavage stage embryos with a dilution series of the purified Au NPs (2.0 mL per well of 0, 0.66, 1.3, 2.4, 5.1, 9.9 and 20 pM or 0, 2.6, 5.1, 9.5, 20, 39 and 78 μg/mL) in 24-well plates (4 embryos/well) for 120 h. We conducted the control experiments by incubating the embryos (4 embryos per well) with egg water alone (in the absence of NPs) and the supernatants collected from washing Au NPs (dispersed in egg water with the same dilution factors as those prepared for Au NPs in egg water) in two rows of the same well plate as those embryos incubated with the NPs. Four replicates (three additional sets of four embryos in each given concentration in each well) were performed simultaneously. Thus, the 16 embryos in three wells for each concentration and control were studied for each run of the experiment. Each experiment was carried out at least three times and a total minimal number of 48 embryos (12 replicates of 4 embryos for each concentration) were studied for each NP concentration and each control experiment to gain representative statistics (Fig. 18).

The microwell plates were incubated in a water bath at 28.5°C under dark for 120 h. The developing embryos in the plates were imaged at 2, 24, 48, 72, 96, and 120 hpf using an inverted microscope (Zeiss Axiovert) equipped with a CCD camera (CoolSnap, Roper Scientific).

**Quantitative imaging of single Au NPs embedded in individual zebrafish**

The cleavage stage embryos were incubated with given concentrations (0 - 20 pM) of the Au NPs continuously (chronically) for 120 h, and developed to normal or deformed zebrafish. We then rinsed the zebrafish with DI water to remove any external NPs, fixed the zebrafish using a tissue processor (Microm
STP-120 Spin, Thermo Fisher Scientific), and sectioned them to prepare ultrathin tissue samples (1 - 4 μm thickness) using a microtome (HM360 rotary microtome, Thermo Fisher Scientific), as we described previously.53, 57-60, 85 The number and sizes of individual Au NPs embedded in the tissues of interest were quantitatively determined using their size-dependent LSPR spectra acquired by DFOMS-MSIS (Figs. 19 - 20). A minimum of three slices of each given tissue were analyzed for each measurement.

**Statistical analysis**

For characterization of sizes, shapes, LSPR spectra, and stability of single Au NPs, we studied 300 individual Au NPs for each sample with a minimum of 100 NPs for each measurement. For real-time imaging of transport and diffusion of single Au NPs into and in embryos, we investigated a minimum of 12 embryos for each given concentration with 4 embryos per measurement. For each NP concentration and each control experiment to study dose-dependent effects of the Au NPs on embryonic development, we characterized a total number of minimal 48 embryos with 12 replicates of 4 embryos per measurement.

Using conventional statistical analysis method (ANOVA), we analyzed the differences of the embryos that developed to normal or deformed zebrafish, or became dead, as they were incubated with various concentrations of the Au NPs (0 - 20 pM), and found insignificant dose-dependent nanotoxicity with a confidence level of 95% (P = 0.05). Notably, we characterized the effects of Au NPs on embryonic development at single embryo and single NP resolution. These approaches enable us to observe the rare events of interest, which otherwise would be buried under ensemble measurements. The study of large number of single Au NPs and single embryos offers sufficient statistics to depict the ensemble properties of NPs and their effects on embryonic development at single NP and single embryo resolutions.
CHAPTER IV
DESIGN OF PLASMONIC NANOPARTICLE PROBES FOR STUDY OF SIZE- AND DOSE-DEPENDENT DEVELOPMENTAL PHENOTYPES AND NANOTOXICITY IN ZEBRAFISH EMBRYOS

INTRODUCTION

With tiny sizes and exceptional surface properties, nanomaterials could enter living organisms non-invasively, interact with biological structures specifically, and create unique effects upon their fates and functions. They can serve as imaging probes for study of vital biological pathways in vivo, and as smart drug delivery vehicles and novel medicines for effective therapy. Thus, it is essential to study the dependence of their biological effects upon their physicochemical properties (sizes, doses, chemical compositions) in order to rationally design their biological functions.

Noble metal nanoparticles, especially Ag NPs, possess distinctive plasmonic optical properties and high Rayleigh scattering. Unlike fluorophores or quantum dots, single Ag NPs resist photobleaching and photoblinking. Their localized surface plasmon resonance (LSPR) spectra highly depend upon their physicochemical properties and surrounding environments. These distinctive optical properties enable them to serve as photostable imaging probes for real-time study of living cells and embryos and dynamic events of interest for a long period of time.

Zebrafish have been widely used as a vertebrate model organism for study of therapeutic effects of drug molecules, environmental effects of toxic chemicals, and embryological development. Zebrafish embryos are optically transparent and develop outside their mothers, enabling direct observation of embryonic development in vivo. Their small size, short breeding cycle, and wealthy information for molecular genetic manipulation, make it a superior model organism over others (mouse, rats, human). Furthermore,
the majority of their developmental mutations have close counterparts in other vertebrates,\textsuperscript{64, 96-98} suggesting that it can effectively serve as a model for understanding the developmental processes of higher organisms, including humans.

Currently, conventional ensemble toxicology methods and \textit{in vitro} cell assays are widely used to study the cytotoxic effects of nanomaterials.\textsuperscript{8, 41-51} Single types of the cultured cells are studied individually, which can miss vital and specific cell-to-cell interactions. In contrast, embryos enable one to simultaneously study the effects of NPs upon a wide variety of cells and detect all related pathways, including reactive oxygen species formation, engagement of apoptotic pathways and disruption of regulatory signaling in the developing embryos.\textsuperscript{28, 50, 51, 93} Furthermore, massive amount of embryos can be generated rapidly (overnight) at very low cost. Thus, they can serve as ultrasensitive and high-throughput \textit{in vivo} assays to study developmental processes upon their exposure to nanomaterials, and to characterize their effects on embryonic development.\textsuperscript{53, 57-60, 85}

Although several studies have used zebrafish embryos to study nanotoxicity,\textsuperscript{53, 57-60, 85, 99, 100} their effectiveness as \textit{in vivo} assays has not yet been fully validated. In our previous studies, we selected cleavage stage embryos and exposed them to various sized Ag or Au NPs chronically for 120 h, and found that the embryos died or developed to deformed zebrafish in a dose-, size-, surface charge-, and chemical-dependent manners.\textsuperscript{53, 57-60, 85} However, none of these studies can determine whether the NPs incite stage-specific abnormalities. In this study, we select cleavage, early gastrulation, early segmentation, late segmentation, and hatching stage embryos (five vital developmental checkpoints), acutely expose them to the purified and stable Ag NPs (97 ± 13 nm) for only 2 h, and characterize their development in egg water over 120 h. This study aims to determine embryonic developmental stage-dependent effects of the NPs and to identify the most sensitive embryos for screening
biocompatibility and toxicity of nanomaterials and vital embryonic developmental stages for further analysis.

RESULTS AND DISCUSSION

Synthesis and characterization of purified and stable Ag NPs (97 ± 13 nm)

We have synthesized and purified the Ag NPs as described previously.\textsuperscript{60,82} The TEM image and histogram of size distribution of single Ag NPs show the polygon NPs with average sizes of 97 ± 13 nm, ranging from 72 to 147 nm with aspect ratios of 0.97 - 3.9 (Figs. 22A - B). The dark-field optical images of single Ag NPs and their LSPR spectra (Figs. 22C - D) show individual plasmonic green, yellow and red NPs, with $\lambda_{\text{max}}$ (FWHM), of 565 (146), 585 (129), and 615 (132) nm, respectively.

To characterize the solubility and stability (non-aggregation) of the Ag NPs suspended in egg water over 120 h at 28.5°C, we determine their number, concentrations and sizes over time using DFOMS, UV-vis spectroscopy and DLS, respectively. The results show that the number of NPs suspended in egg water remains essentially unchanged over 120 h (Fig. 23A). Their UV-vis absorption spectra show that their peak absorbance of 2.1 at 459 nm (FWHM = 100 nm) and the absorbance of 1.9 at a shoulder peak of 400 nm (FWHM = 76) remain unchanged (Fig. 23B). Their sizes in egg water measured by DLS at 0 and 120 h are 89 ± 17 nm and 92 ± 19 nm, respectively. The results indicate that their sizes remain essentially unchanged over 120 h (Fig. 23C). Taken together, the results (Fig. 23) show that the Ag NPs (24 pM) are stable (non-aggregated) and fully suspended in egg water over 120 h.

It is crucial to use purified and stable NPs to study their dose, size and stage-dependent effects on embryonic development, because the unpurified NPs could contain undetermined amounts of various chemicals involved in NP synthesis, which can cause various toxic effects and lead to inconclusive or
Figure 22. Characterization of sizes, shapes and plasmonic optical properties of single Ag NPs suspended in egg water.

(A) HR-TEM images show polygon shaped NPs. (B) Histogram of the size distribution of single Ag NPs measured by HR-TEM showing an average size of 97 ± 13 nm. The size of each NP is determined by averaging its length and width. (C) Dark-field optical image of single Ag NPs showing individual green, yellow, and red-orange NPs. (D) LSPR spectra of single Ag NPs in (C) show $\lambda_{\text{max}}$ (FWHM) values of (a) 565 (146), (b) 585 (129), and (c) 615 nm (132). Scale bars = 100 nm in (A) and 2 µm in (C). The scale bar in (C) shows the distances among individual NPs, but not their sizes because of the optical diffraction limit.
Figure 23. Study of the stability (non-aggregation) and solubility of the Ag NPs dispersed in egg water.

(A) The average number of single Ag NPs acquired by DFOMS over time shows 167 ± 3, 171 ± 8, 167 ± 1, 172 ± 10, 180 ± 11, and 179 ± 3 NPs per image at 0, 24, 48, 72, 96, and 120 h, respectively. (B) UV-vis absorption spectra of Ag NPs in egg water at 28.5°C for (a) 0 and (b) 120 h show that the peak absorbance of 2.1 at 459 nm (FWHM of 100 nm) and the absorbance of 1.9 at a shoulder peak of 400 nm (FWHM of 76 nm) remain essentially unchanged over 120 h. (C) Histograms of size distributions of the NPs in egg water measured by DLS show that their average sizes are 89 ± 17 and 92 ± 19 nm after incubation for (a) 0 and (b) 120 h, respectively.
irreproducible results. Furthermore, if the NPs were aggregated during their exposure, their sizes could increase and their concentrations could decrease over time, making the study of dose- and size-dependent nanotoxicity unreliable.

**Specific embryonic developmental stages**

The five embryonic developmental stages (Fig. 24): (A) cleavage (2 hours-post-fertilization, hpf), (B) early gastrula (6 hpf), (C) early segmentation (12 hpf), (D) late segmentation (21 hpf), and (E) hatching stage (48 hpf), are vital checkpoints of embryonic development. Here we name them (A - E) as stage I-V embryos for easy comparison, respectively. We incubate each given stage embryos with given doses of the Ag NPs for only 2 h (acute treatment), thoroughly rinse them with egg water to remove external NPs, and then incubate and monitor their development in egg water at 28.5°C over 120 h until they fully develop.

Upon their 2 h exposure to the NPs, cleavage stage embryos (stage I: 2 - 4 hpf) undergo rapid cellular division and embryonic pattern formation to set forth for the development of different organs. Gastrula stage embryos (stage II: 6 - 8 hpf) undergo cell movements and migrations to rearrange two largely separable tissues, form germ layers and establish the early organ systems. Early segmentation stage embryos (stage III: 12 - 14 hpf) begin morphological cell differentiation, morphological movements, somitogenesis and notochord formation, which is important for proper development of the axial skeleton, the vertebrate spinal column, and the skeletal muscle. Late segmentation stage embryos (stage IV: 21 - 23 hpf) undergo development of the circulatory system and the formation of heart. The hatching stage embryos (stage V: 48 - 50 hpf) are completing their embryonic development and all related organs have been formed.
Dose- and stage-dependent biocompatibility and toxicity of Ag NPs

We expose each given stage embryos to a dilution series of the Ag NPs (0 - 24 pM, 0 - 72 µg/mL) for 2 h and monitor their development in egg water over 120 h. We characterize the molar concentrations of NPs in egg water, as we described previously.\(^5\), \(^3\), \(^5\), \(^8\) Note that unlike weight/volume concentrations, molar concentrations of the NPs are proportional to their number and surface area, which are more appropriate to describe their surface-dependent effects, and are used throughout our studies.\(^5\), \(^3\), \(^5\), \(^7\), \(^6\), \(^0\), \(^8\) The results show that the NPs incite dose- and stage-dependent nanotoxicity toward embryonic development (Fig. 25). The earlier developmental stages of embryos are more sensitive to the effect of the NPs than the later stages of embryos. Furthermore, the percentages of embryos that develop normally decrease and the percentages of dead embryos increase, as the NP concentration increases from 0 to 24 pM.

Upon the exposure of stage I embryos to 2 pM Ag NPs (Fig. 25A), 82 ± 11% of the embryos develop normally, 16 ± 9% of them die and 2 ± 4% of them develop abnormally. As the NP concentration increases, the percentages of
normally developed zebrafish decrease drastically. At 4 pM NPs, 42 ± 10% of embryos develop normally, 48 ± 15% of them die and 11 ± 7% of them develop abnormally. At 8 pM NPs, only 22 ± 10% of the embryos develop normally, 78 ± 10% of them die and none of them develop abnormally. At 16 pM NPs, only 2 ± 4% of the embryos develop normally and 98 ± 4% of them die. At 24 pM NPs, all embryos die.

For stage II embryos (Fig. 25B), the percentages of embryos that develop normally decrease more gradually than stage I embryos as the NP concentration increases. At 2 pM NPs, 59 ± 11% of the embryos develop normally, 36 ± 10% of them die, and 5 ± 5% of them develop abnormally. At 4 pM NPs, 56 ± 15% of embryos develop normally, 39 ± 17% of them die, and 4 ± 4% of them develop abnormally. At 8 pM NPs, 32 ± 14% of the embryos develop normally, 63 ± 11% of them die, and 5 ± 4% of them develop abnormally. At 24 pM NPs, 13 ± 12% of the embryos still develop normally, 82 ± 14% of them die, and 5 ± 4% of them develop abnormally.

For stage III embryos (Fig. 25C), they either develop normally or die, and few develop abnormally upon their acute exposure to the NPs. The percentages of embryos that develop normally decrease nearly as rapidly as the stage I embryos as the NP concentration increases. At 2 pM NPs, 98 ± 4% of the embryos develop normally, only 2 ± 4% of them die, and none of them develop abnormally. At 4 pM NPs, 76 ± 15% of embryos develop normally and 24 ± 15% of them die. At 8 pM NPs, only 25 ± 11% of the embryos develop normally, 72 ± 9% of them die, and 2 ± 4% of them develop abnormally. At 16 pM NPs, only 2 ± 4% of the embryos develop normally, 98 ± 4% of them die, and none of them develop abnormally. At 24 pM NPs, all embryos die.

For stage IV embryos (Fig. 25D), they either develop normally or die, and few develop abnormally upon their acute exposure to the NPs, as those observed in stage III embryos. The percentages of embryos that develop normally decrease slightly more gradually than the stage III embryos as the NP
concentration increases. At 2 pM NPs, all embryos develop normally. At 4 pM NPs, 88 ± 13% of embryos develop normally and 8 ± 14% of them die. At 8 pM NPs, only 25 ± 13% of embryos develop normally, 75 ± 13% of them die and 4 ± 7% of them develop abnormally. At 24 pM NPs, only 4 ± 7% of the embryos develop normally, 96 ± 7% of them die and none of them develop abnormally.

For stage V embryos (Fig. 25E), they either develop normally or die, and few develop abnormally upon their acute exposure to the NPs, similar to those observed in stages III - IV embryos. The percentages of embryos that develop normally decrease much more gradually than the stage IV embryos as the NP concentration increases. At 2 pM NPs, 94 ± 6% of embryos develop normally, 4 ± 3% of them die and 2 ± 3% of them develop abnormally. At 4 pM NPs, 88 ± 16% of embryos develop normally, and 12 ± 16% of them die, none of them develop abnormally. At 8 pM NPs, only 50 ± 13% of embryos develop normally, 44 ± 6% of them die and 6 ± 6% of them develop abnormally. At 24 pM NPs, 24 ± 10% of the embryos still develop normally, and 76 ± 10% of them die.

We conduct two control experiments simultaneously by incubating stage I - V embryos with egg water alone (blank control) and supernatant collected from the last washing of the NPs for 2 h, and monitoring the embryonic development in egg water over 120 h. Similar to those of blank control, 94 - 100% of embryos develop normally, and none of embryos develop to deformed zebrafish, and they are independent upon the concentrations (doses) of supernatant (Fig. 25F). The control experiments with the supernatants enable us to eliminate potential toxic effects of trace chemicals (e.g., Ag⁺, citrate) resulted from the Ag NP synthesis or their potential degradation over time, and to validate that the observed toxic effects of the NPs on embryonic development are attributed to the NPs.

Taken together, these interesting findings show unambiguous stage and dose dependent toxic effect of the Ag NPs upon embryonic development. The results show that stage I embryos are most sensitive to the toxic effects of the NPs with the lowest critical concentration (3.5 pM), which is followed closely by
Figure 25. Study of dose- and stage-dependent effects of the Ag NPs on embryonic development.

Histograms show the percentages of stage I - V embryos that have been incubated with (A - E) Ag NPs or (F) the supernatant for 2 h that develop to normal and deformed zebrafish or die in egg water over 120 hpf.
stage II (4 pM), stages III and IV embryos (6 pM), while stage V embryos are most resistant to the NPs with a critical concentration of 8 pM.

**Stage-specific embryonic phenotypes**

Upon the exposure of stages I and II embryos to the NPs for 2 h, some of the embryos develop to deformed zebrafish with seven types of abnormalities: (a) finfold abnormality and tail/spinal cord flexure; (b) cardiac malformations/edema, yolk sac edema, finfold abnormality and tail flexure; (c) eye abnormality, cardiac malformations/edema, yolk sac edema, finfold abnormality, and tail flexure; and (d) no eyes, head abnormality, cardiac malformations/edema, yolk sac edema, finfold abnormality, and tail flexure (Figs. 26A - B), respectively. Notably, head abnormality with an enlarged brain and no eyes (Fig. 26d) are only observed in the treated stages I and II embryos to 4 and 24 pM NPs, respectively. Multiple abnormalities are observed in the same zebrafish.

Interestingly, we observe only five types of abnormalities (a - c) (Figs. 26C - D), in the treated stages III and IV embryos. Furthermore, we observe only four types of abnormalities (a - b) (Fig. 26E), in the treated stage V embryos. Note, the no eyes and head abnormality deformations were not observed in stages III – V. Similar to those observed in stages I and II embryos, multiple abnormalities are also in same zebrafish.

Finfold abnormalities without fin rays and tissue structures are one type of shared abnormalities of deformed zebrafish developed from the treated stages I - V embryos. In normally developed zebrafish (Fig. 26F), the finfold is a clear, thin membrane, composed of several epidermal layers that surround the entire trunk region. In deformed zebrafish, the finfold structure is disordered and in some cases non-existent, which are typically accompanied by tail flexures (Fig. 26a - a). Abnormal tail/spinal cord flexures are the other shared defects of deformed zebrafish developed from the treated stages I - V embryos. In normally developed zebrafish (Fig. 26F), the notochord and spinal cord develop straight to
Figure 26. Optical images of deformed and normal zebrafish and dead embryos.

Optical images of (A - E) deformed zebrafish were observed after stage I - V embryos had been incubated with the Ag NPs for 2 h and developed in egg water over 120 hpf. Multiple types of abnormalities occur in the same zebrafish: (a) finfold abnormality and tail flexure, (b) cardiac malformation/edema, yolk sac edema, finfold abnormality, and tail/spinal cord flexure, (c) eye abnormality, cardiac malformation/edema, yolk sac edema, finfold abnormality, and tail/spinal cord flexure, and (d) no eye defect, head deformity, cardiac malformation/edema, yolk sac edema, finfold abnormality, and tail/spinal cord flexure (the most severe and rare deformities). Optical image of (F) normal and (G) dead zebrafish. Scale bars = 500 μm.
the posterior-most tip of the tail. However, in the deformed zebrafish, the tail regions are flexed to some extent. In the severest cases, the tail is completely bent, kinked and truncated (Fig. 26a - d).

Cardiac malformations/edema is another type of shared abnormality of the deformed zebrafish developed from the treated stages I - V embryos. In contrast to normally developed zebrafish (Fig. 26F), the pericardial sac region of deformed zebrafish developed from the treated embryos is swollen and enlarged. In the severest cases, the pericardial sac is extremely large and the size of cardiac ventricle is reduced (Fig. 26b - d).

Yolk sac edema is another type of shared abnormality of deformed zebrafish developed from the treated stages I - V embryos. In normally developed zebrafish (Fig. 26F), the yolk sac region is a bulbous area containing yolk that provides nutrients to the developing embryos and it shrinks during the later developmental stages. In contrast, the deformed zebrafish show swollen and enlarged yolk sac region (Fig. 26b - d). In some cases, they also display edema of the pericardial sac region. Interestingly, yolk sac edema is accompanied by cardiac malformations/edema, abnormal tail/spinal cord flexures, and finfold abnormalities.

Eye abnormality is observed only in the deformed zebrafish developed from the treated stages I - IV embryos. In normal zebrafish, the eyes are darkly pigmented with a well-developed retina surrounded by a lens (Fig. 26F). In deformed zebrafish, the eyes are either smaller, with abnormal structures and shapes, or without the lens (Fig. 26c - d).

The most rare and severe deformities are observed in deformed zebrafish developed from the treated stages I and II embryos. The head is swollen and enlarged with abnormal shapes and without eye (Fig. 26d). Interestingly, for both stages, these deformities are concomitant with finfold abnormality, tail flexure, cardiac malformation, yolk sac edema, and eye abnormality. These results indicate that developmental events occurring during stage I and II treatments are
the most critical to embryonic development and they are most sensitive to such NPs (97 ± 13 nm).

For stage I embryos (Fig. 27A), finfold abnormalities are the primary defects with accumulation percentage of 32 ± 11% (sum of their percentages observed in all NPs concentrations). The percentage of finfold abnormalities increases from 5% to 26% as the NP concentration increases from 2 to 4 pM. The tail/spinal cord flexures are the secondary defects with 21 ± 7%. Cardiac malformations/edema and yolk sac edema are the tertiary abnormalities, each with 16 ± 7%. The eye abnormalities, head edema and no eye defect are the rarely observed quaternary defects, each with 5 ± 2%.

For stage II embryos (Fig. 27B), finfold abnormalities and tail/spinal cord flexures are the primary defects of deformed zebrafish, each with 25 ± 0%, which is closely followed by cardiac malformations/edema and yolk sac edema as secondary and tertiary abnormalities of 20 ± 1% and 18 ± 1%, respectively. The eye abnormalities, head edema and no eye defect are the quaternary defects of 8 ± 1%, 3% and 4%, respectively.

For stages III and IV embryos (Figs. 27C - D), finfold abnormalities, tail/spinal cord flexures, cardiac malformations/edema, yolk sac edema, and eye abnormalities are the primary defects, each with 20 ± 9%. In contrast, for stage V embryos, cardiac malformations/edema and yolk sac edema are the primary defects, each with 30 ± 15%, while finfold abnormalities and tail/spinal cord flexures are the secondary defects, each with 20 ± 8%.

For stages I - II embryos, all organ structures (e.g., finfold, tail, cardiac, eye, and head) are not yet developed. However, cleavage stage embryos (2 - 4 hpf) undergo rapid cell division and embryonic pattern formation. The gastrula stage embryos (6 - 8 hpf) undergo substantial changes in cell shape, adhesion and migration. Gene regulatory systems are actively programming the embryonic tissues for their eventual roles in development. These earliest determinative events to generate the organ structures are ongoing. Therefore,
Figure 27. Characterization of dose- and stage-dependent embryonic developmental abnormalities.

(A - E) Histograms of the percentages of the given stage embryos, which have been incubated with given concentrations of the NPs for 2 h and develop to deformed zebrafish in egg water at 120 hpf with (a) finfold abnormality, (b) tail/spinal cord flexure, (c) cardiac malformations/edema, (d) yolk sac edema, (e) eye abnormality, (f) head abnormality, and (g) no eye defect for (A) stage I, (B) stage II, (C) stage III, (D) stage IV, and (E) stage V embryos.
the defects we observed 4 days later (120 hpf) must have been downstream effects of disruptions of the earliest determinative events, which include gene transcription, cell signaling, cell - cell communication, cell division and migration. Treatment of stages I and II embryos uniquely produces head and no eye defects (Fig. 26d). The occurrence of these defects is notable given that the formation of head and eye structures does not occur until many hours following the exposure, suggesting that the NPs target regulatory molecules during these determinative stages of development.

In contrast, the stage III embryos undergo the differentiation of organ structures and formation of somites and notochord. These structures are important for proper development of the axial skeleton, the vertebrate spinal column, and the skeletal muscle. Thus, the effects of the NPs upon stage III embryos are likely to be more direct, perhaps disrupting protein synthesis and/or cytoskeletal formation required to support finfold and somite formation and their proper organization. Interestingly, the equal percentages of finfold abnormalities, tail/spinal cord flexures, cardiac malformations/edema, yolk sac edema, and eye abnormalities are observed.

For stage IV embryos, the last somites, circulatory system and heart are formed, and the heart is preparing for its first contraction. Similar to stage III embryos, the effects of the NPs upon the developmental abnormalities are likely to be more direct, perhaps disrupting the formation of circulatory system and heart, which leads to the cardiac malformations/edema with extremely large pericardial sac and small cardiac ventricles. Notably, the equal percentages of cardiac malformations/edema, yolk sac edema, eye abnormalities, finfold abnormalities, and tail/spinal cord flexures are observed.

For stage V embryos, all organs required for survival have already been developed. Yet, the heart is newly formed and the yolk sac is still shrinking. Similar to stages III - IV embryos, the effects of the NPs upon their development are likely to be direct, perhaps disrupting the heart function and the shrinking of
the yolk sac, which leads to primary cardiac malformations/edema and yolk sac edema.

Characterization of single Ag NPs embedded in tissues of zebrafish

To determine whether the NPs stay inside the embryos throughout their development upon their 2 h incubation with the embryos, we characterize single Ag NPs embedded in various tissues of zebrafish developed from the given stage of embryos treated with the NPs using DFOMS-MSIS. Longitudinal thin-layer sections (2 - 4 μm thickness) of deformed zebrafish with all types of the deformities show the NPs embedded in its eye (retina), pericardial space (heart), and tail, as characterized by distinctive LSPR spectra of individual Ag NPs (Fig. 28A - C). The red shift of LSPR spectra of individual Ag NPs embedded in the tissues with much larger FWHM could be attributed to the changes of their surrounding environments and surface properties (e.g., dielectric constant of embedded medium) which differ from egg water (Fig. 22D), and/or the increase of their sizes.

Notably, we did not observe the blue-shift of the LSPR spectra of single Ag NPs embedded in the tissues of zebrafish. The results show that the Ag NPs are not degraded (broken apart or dissolved) during their incubation with the embryos. If the Ag NPs were degraded, dissolved or broken apart, we would have observed the blue-shift of their LSPR spectra due to the decrease of their sizes. Note, our DFOMS-MSIS is sufficiently sensitive to detect the change of single molecules on the surface of single NPs.

Using the same approaches, we also prepare the thin-layer sections of the normally developed zebrafish from the control experiments (acute exposure of the embryos to egg water or supernatant for 2 h, and fully develop in egg water at 120 hpf) and used DFOMS-MSIS to characterize the scattering spectra of the tissues (Fig. 28D - F). The scattering spectra of the tissues of the eye, heart and tail show broad background (Fig. 28E), which are unlike the distinctive LSPR spectra of single Ag NPs (Fig. 28B).
Figure 28. Imaging and characterization of individual Ag NPs embedded in the tissues of deformed and normal zebrafish using DFOMS-MSIS.

Optical image of thin-layer longitudinal section of fixed (A) deformed zebrafish with five types of deformities and (D) normal zebrafish. (C and F) Zoomed-in optical images of the tissue sections of (a – c) highlighted in (A) and (D), respectively: (a) eye (retina), (b) pericardial space, and (c) tail. (B) LSPR spectra of individual Ag NPs as circled in (C) show distinctive $\lambda_{\text{max}}$ (FWHM) values of (a) 567 (176), (b) 688 (185), and (c) 759 nm (179). (E) Scattering intensity of the tissues of normal zebrafish in (F) showing the background (non-distinctive plasmonic colors). Scale bars = 250 µm in (A, D), 5 µm in (C) and 30 µm in (F).
Taken together, these results show that such large Ag NPs (97 ± 13 nm) enter the developing embryos during their 2 h incubation, and the NPs remain inside the embryos throughout their development. LSPR spectra of single Ag NPs embedded in the tissues can be used to unambiguously identify and determine single Ag NPs in the tissues (Fig. 28B); even though the optical image themselves (Fig. 28A and C) cannot clearly display the NPs because of the high background scattering of the tissues. Thus, the results (Fig. 28) clearly show that the LSPR spectra of single Ag NPs enable us to qualitatively and quantitatively detect single Ag NPs embedded in the tissues of interest. Unlike any conventional methods (e.g., ICP-MS or atomic absorption spectroscopy), DFOMS-MSIS offers single NP sensitivity, the sub-micrometer spatial resolution, simple sample preparation, and high-throughput capability for in situ analysis of single NPs embedded in the tissues. Furthermore, DFOMS-MSIS is a non-invasive method, and can preserve tissue samples, especially rare phenotypes, for further analysis.

SUMMARY

In summary, we have synthesized and characterized stable and purified Ag NPs (97 ±13 nm). We found that the Ag NPs stay inside the embryos throughout their development (120 h) upon their 2 h incubation with each given stage of embryos. Notably, the Ag NPs incite distinctive stage-dependent toxicity and create stage-specific phenotypes. The cleavage stage embryos are most sensitive to the toxic effect of the NPs with the lowest critical concentration of NPs (3.5 pM), which is followed by gastrula stage embryos (4 pM), and early and late segmentation stage embryos (6 pM), while hatching embryos are most resistant to the NPs (8 pM). Cleavage stage and gastrula stage embryos develop to seven types of abnormalities, including head abnormality and no eye defects, while early and late segmentation stage embryos develop to only five types of abnormalities without head and no eye deformities, and hatching
embryos develops to four abnormalities without eye and head deformities. The observed abnormalities developed from the treated cleavage and gastrula stage embryos suggest that NPs disrupt early determinative events (e.g., cell signaling and gene transcription) and create downstream effects upon embryonic development. The observed abnormalities developed from the treated segmentation stage and hatching embryos suggest direct effects of the NPs upon embryonic development. The disruption of the early determinative events is more effective to create abnormalities and they are more deadly. The toxic effects of NPs on embryonic development increase with their concentration, showing an unclear threshold, which indicates that Ag NPs differ from conventional chemicals. In comparison with our study of the smaller Ag NPs (13.1 ± 2.5 nm), we found striking size-, stage- and dose-dependent toxic effect of Ag NPs upon embryonic development, suggesting that the NPs can create specific target in embryonic development by tuning their sizes and doses.

METHODS

Reagents and supplies

All reagents and chemicals were purchased from Sigma and used as received. The nanopure water (18 MΩ water, Barnstead) was used to prepare all solutions and rinse glassware.

Synthesis and characterization of purified and stable Ag NPs

The Ag NPs were synthesized and characterized as we described previously. Briefly, sodium citrate (20 mL, 34 mM in deionized (DI) water) was rapidly added into AgNO₃ (500 mL, 1.06 mM in DI water) under stirring and refluxing. The colors of the mixture turned from colorless to straw yellow, then opaque yellow, and finally muddy yellow, during the 95 min stirring and refluxing. We stopped the heating, continued refluxing and stirring the solution until it was cooled to room temperature, then immediately filtered it using 0.22 μm filters.
The NPs were washed three times with nanopure DI water using centrifugation to remove the chemicals involved in NP synthesis. The supernatants of NP solution after the third washing were collected for control experiments to study the effect of trace chemicals involved in NP synthesis on the embryonic development.

The concentrations, optical properties, and sizes of NPs were characterized using UV-vis spectroscopy (Hitachi U2010), dark-field optical microscopy and spectroscopy (DFOMS), dynamic light scattering (DLS, Nicomp 380ZLS particle sizing system), and high-resolution transmission electron microscopy (HR-TEM, JEOL, JEM-2100 F). We have fully described our DFOMS and MSIS for real-time imaging and spectroscopic characterization of single NPs in solutions, single live cells and single embryos, and for single molecule detection.20-22, 52-54, 56-60, 79-85

Breeding and monitoring of zebrafish embryos

Wild type adult zebrafish (Aquatic Ecosystems) were housed in a stand-alone system, maintained, bred, and collected, as described previously.53, 54, 57-60, 85-86 Briefly, male and female zebrafish at a ratio of 1 to 2 were placed in a clean 10 gallon breeding tank at 28.5°C, and a light (14 h) - dark (10 h) cycle was used to trigger breeding and fertilization of embryos. Each given developmental stage embryos were collected, transferred into a petri dish containing egg water (1.0 mM NaCl in DI water), and washed three times with egg water to remove the surrounding debris. All experiments involving embryos and zebrafish were conducted in compliance with the IACUC guidelines (protocol #11-030).

Quantitative study of dose and stage-dependent toxicity of the Ag NPs

The given stage embryos were incubated with a dilution series of the Ag NPs (0, 2, 4, 8, 16, and 24 pM) or (0, 6.03, 12.06, 24.12, 48.24, and 72.36 mg/L) in a 24-well plate with four embryos per well at 28.5°C (water bath) for 2 h (acute treatment). The embryos were then thoroughly rinsed with egg water to remove
external NPs, and re-placed in the wells of a new 24-well plate containing egg water with 4 embryos per well. As control experiments, the embryos that had been incubated with egg water (blank control) or supernatant of the last washing of the NPs (in the absence of NPs) for 2 h were rinsed and placed in two rows of the same well plates, aiming to determine any potential effects of trace chemicals from NP synthesis upon embryonic development.

The embryos in the well plates were incubated in the water bath at 28.5°C, and directly imaged at room temperature every 24 h using an inverted microscope (Zeiss Axiovert) equipped with a CCD camera (CoolSnap, Roper Scientific) and a digital color camera (Sony). Each experiment was carried out 4 times. The 48 embryos at each given stage (4 repeat experiments with 3 replicates of 4 embryos per measurement) were studied for each given NP concentration to gain representative statistics.

**Imaging and characterization of Ag NPs embedded inside zebrafish**

The given stage embryos were incubated with given concentrations of the Ag NPs (0 - 24 pM) for 2 h, thoroughly rinsed with egg water, and developed to normal or deformed zebrafish in egg water at 120 hpf. The normal and deformed zebrafish were rinsed with DI water to remove any external NPs, fixed using a tissue processor (Microm STP-120 Spin, Thermo Fisher Scientific), then embedded with paraffin using a tissue embedding center (Shandon Histocentre™ 3 Embedding Center), and finally sectioned to prepare thin-layer tissue samples (2 - 4 μm thickness) using a microtome (HM360 rotary microtome, Thermo Fisher Scientific), as we described previously.53, 58, 60, 85 Individual Ag NPs embedded in the tissues of interest was determined using their LSPR spectra acquired by DFOMS-MSIS.

**Statistical analysis**

For characterization of sizes, shapes, LSPR spectra, and stability (non-aggregation) of single Ag NPs, 300 individual NPs were studied for each sample
with a minimum of 100 NPs per measurement. For study of dose- and stage-dependent biocompatibility and toxicity of Ag NPs toward embryonic development, 48 embryos at each given stage were studied for each NP concentration with 4 repeating measurements, and each measurement has 12 embryos with 4 embryos per well. We used statistical analysis (ANOVA, Tukey's, SigmaStat 3.5, with $P = 0.05$) to examine significant differences among our observations of normal, dead and deformed zebrafish in different concentrations of Ag NPs and different stage of embryos.
CHAPTER V

NOVEL GREEN METHODS FOR STUDY OF EMBRYONIC STEM CELLS

INTRODUCTION

Embryonic stem cells (ESCs) are pluripotent cells that can self-renew indefinitely, differentiate and develop into various specialized cell types with specific functions, offering possibilities of using ESC-based therapies to treat an array of diseases and disorders. However, enormous challenges must be overcome in order to realize such incredible therapeutic potentials. For example, it remains essentially unknown how ESCs differentiate, how one can effectively and specifically guide their differentiation, and how one can design simple and effective techniques to culture and sustain undifferentiated ESCs over time. An effective means to culture and maintain ESCs in undifferentiated states will lay the foundation to address the critical questions about the differentiation of ESCs.

Currently, ESCs are cultured on a layer of feeder cells, which are viable and growth-arrested (or mitotically inactive) and provide required growth factors to sustain and support the self-renewal of the ESCs. The types of feeder cells vary with the species of ESCs. For instance, human ESCs (e.g., BG01V) typically use either human (e.g., fibroblasts) or mouse cells (e.g., CF-1) as feeder cells, and mouse ESCs (e.g., C57Bl/6) use mouse cells as feeder cells (fibroblasts). Zebrafish ESCs use rainbow trout cells as feeder cells. Therefore, specific feeder cells are essential for the proliferation of undifferentiated ESCs.

To date, only two methods, gamma (γ)-radiation or chemical inactivation using mitomycin-C, have been used to prepare feeder cells. γ-radiation requires expensive equipment with specific radiation sources and the sources must be properly calibrated in order to effectively prepare feeder cells. Note that distance and shielding factors can dramatically affect radiation doses,
leading to irreproducible results. Furthermore, the feeder cells prepared using γ-radiation often become apoptotic with time and detach from the surface after weeks of culture, which will undoubtedly affect the growth of ESCs. Mitomycin-C (a chemotherapeutic agent) arrests cell proliferation by inhibiting the separation of dsDNA during cell replication. Even though, mitomycin-C treatment is cheaper and faster than γ-radiation, it can metabolically alter the feeder cells and inhibit their expression of specific ligands or cytokines required to culture and sustain undifferentiated ESCs. The deficiencies of both methods demand the development of new and effective means to prepare high-quality feeder cells for the culture of ESCs.

Recent studies show that ultrashort electric pulses (e.g., 10 ns) can penetrate inside the cells and induce intracellular responses while maintaining viability of cells. The intracellular structures and viability of cells depend on the number and electric field strength (E) of 10-ns electric pulses (10nsEPs), showing the possibility of tuning them to prepare a wide variety of viable growth-arrested cells. Conventional electroporators, offer 50-µs electric pulses (50µsEPs), have been widely used to deliver genes into living cells. They are inexpensive and widely available in various research laboratories. In this study, we used 10nsEPs or 50µsEPs to successfully prepare viable and growth-arrested feeder cells to culture and sustain undifferentiated zebrafish ESCs over time. To our knowledge, this is the first time that pulsed electric fields (PEFs) have been used to prepare the growth-arrested feeder cells for culturing ESCs.

RESULTS AND DISCUSSION

Using PEFs to generate growth-arrested feeder cells

We exposed the cell (RTS34st) suspension in electroporation cuvettes to 25 sequential 10nsEPs at 40 kV/cm (1-s pulse interval) and cultured the exposed cells in growth medium over time. The growth curves of the cells (Fig. 29A) show that their number decreases slightly and then remains unchanged over time after
the third day of culture. In contrast, the number of the unexposed cells (without being exposed to 10nsEPs) cultured in the growth medium continues to increase with time (Fig. 29A). Optical images of the cells on the surface of the flasks illustrate that the exposed cells elongate, but do not replicate and divide over time (Fig. 29B), while the unexposed cells grow and divide over time (Fig. 29C).

We determined the viability of the cells cultured in the medium for 14 days using an Alamar Blue assay. Alamar blue (resazurin, $\lambda_{\text{max}}$ of absorption spectrum at 600 nm) is highly permeable to live cells and is non-cytotoxic.\cite{118,119} It can be reduced to resorufin (pink, $\lambda_{\text{max}} = 570$ nm) by reductases in living cells, while it remains blue in dead cells. Therefore, Alamar blue can serve as a colorimetric assay to determine cellular viability in real-time.\cite{118,119} We assayed the suspensions in the flasks (Fig. 29D) containing the medium alone (control, Fig. 29D: i), unexposed (Fig. 29D: ii) and exposed cells (Fig. 29D: iii) cultured for 14 days using Alamar blue. The medium alone remained the blue, while the unexposed and exposed cells cultured for 14 days turned from blue to pink, showing that both cells were alive. These results demonstrate that 10nsEPs can be used to prepare viable growth-arrested cells, suggesting that 10nsEPs penetrate into the cells and inactivate mitotic processes by mechanisms, such as disruption of DNA replication or cell cycle check-points, but do not disrupt cellular viability.

The growth curves of the cells exposed to 25 sequential 10nsEPs of 150 (Fig. 30A) or 25 kV/cm (Fig. 30B) show the number of cells decreases and increases with time, respectively. Their optical images (Fig. 30A: b – d) illustrate that the cells exposed to 10nsEPs of 150 kV/cm show abnormal spherical morphologies and do not elongate over time. Note that the cells with spherical shape are generally apoptotic, due to the loss of membrane integrity.\cite{120} In contrast, the cells exposed to the 25 kV/cm 10nsEPs elongated, grew and divided over time. The results show that the effects of 10nsEPs on the cellular functions are highly dependent upon their E. We selected an approximate pulse number and E of 10nsEPs to prepare viable growth-arrested cells based upon
Figure 29. Preparation and characterization of viable growth-arrested feeder cells.

(A) The growth curves of cells that had been exposed to 25 sequential 10nsEPs of 40 kV/cm with 1-s pulse interval (diamonds) show that the number of cells decreases and then remains constant after day 3 of culture; for unexposed cells (control, squares), the number of the cells increases with time. Optical images of the (B) exposed and (C) unexposed cells in (A) cultured on day (a) 1, (b) 7 and (c) 14, show that the number of the cells remains nearly unchanged and that the cells grow with time, respectively. (D) Photo of the flasks containing Alamar blue with (i) medium alone, (ii) unexposed cells in (C), and (iii) exposed cells cultured on day 14 in (B), show blue color in (i), and pink colors in (ii) and (iii), indicating that the cells in (ii) and (iii) are viable. Scale bars = 500 μm.
Figure 30. The effects of electric field strength of 10nsEPs on cell growth.

(a) Growth curves of the cells that had been exposed to 25 sequential 10nsEPs with 1-s pulse interval, at E of (A) 150 and (B) 25 kV/cm; (b – d) their optical images on (b) day 1, (c) day 7, and (d) day 14 of culture. Scale bars = 100 μm.

Using the same approaches, we explored the possibility of using 50μsEPs provided by conventional electroporators that are widely available in research labs and much more user-friendly than 10nsEPs, to prepare the viable growth-arrested cells. The cells exposed to two sequential 50μsEPs of 2.82, 1.78 and 0.78 kV/cm with 5-s pulse interval, were cultured in the medium and imaged over time. The growth curves (Figs. 31A - C: a) show that the number of cells
decreases with time, remains constant and increases with time for the cells that were exposed to two sequential 50μsEPs of 2.82, 1.78 and 0.78 kV/cm, respectively. Their optical images illustrate the growth-arrested cells with abnormal cellular morphology (Fig. 31A: b – d), elongated and undivided cells with normal morphologies (Fig. 31B: b – d), and growth and division of cells with normal morphology (Fig. 31C: b – d). As noted above, the spherical cells are apoptotic, due to the loss of membrane integrity.120

The exposed cells that were cultured for 5 days (Figs. 31A - C) are viable, as determined by Alamar blue assay (Fig. 31D). Notably, the flasks (i - v) in Figure 31D containing Alamar blue with the medium alone (blank control, i), the unexposed cells (positive control, ii), and the cells exposed to the 50μsEPs of 2.82 (iii), 1.78 (iv) and 0.78 kV/cm (v), showed blue, bright pink, dark blue-pink, light pink, and bright pink, respectively. The results show that the 50μsEPs at 2.82 kV/cm affect the cellular viability, while the pulses at 1.78 and 0.78 kV/cm did not. Taken together, these results demonstrate that the two sequential 50μsEPs at 1.78 kV/cm can produce viable growth-arrested cells, which can serve as feeder cells for culture of undifferentiated ESCs. The effects of 50μsEPs on the cellular functions highly depend upon their E, similar to those observed using 10nsEPs (Fig. 30).

**Culture and sustainability of undifferentiated ESCs on the feeder cells**

We used the viable growth-arrested cells prepared using PEFs as described above, as a monolayer of the feeder cells and cultured the zebrafish ESCs on them. We freshly isolated the ESCs from zebrafish embryos and directly cultured them on the monolayer of feeder cells (Fig. 32A) and on the bare surface of wells (Fig. 32B) for 5 days. The feeder cells exposed to two sequential 50μsEPs at 1.78 kV/cm were cultured on the wells of cell plates for 5 days, producing a monolayer of mitotically inactivated cells. Notably, the cell number remained unchanged for additional 6 days of cultures during which ESCs were cultured on them (Fig. 32C).
Figure 31. Effects of electric field strength of 50µsEPs on growth and viability of cells.

(a) Growth curves of the cells that had been exposed to two sequential 50µsEPs with 5-s pulse interval at E of (A) 2.82, (B) 1.78 and (C) 0.78 kV/cm; (b – d) their optical images on (b) day 1, (c) day 3, and (d) day 5. (D) Photo of the flasks containing Alamar blue with: (i) medium alone, (ii) the unexposed cells, (iii – v) the cells on day 6 in (A – C), respectively, show blue color in (i) and pink colors in (ii – v), indicating that the cells in (ii – v) are viable. Scale bars = 100 µm.
Figure 32. Characterization of the morphologies of undifferentiated ESCs cultured on the growth-arrested feeder cells prepared using PEFs.

Optical images of ESCs cultured on (A) the monolayer of feeder cells, and (B) on a bare surface of wells without the feeder cells on (a) day 1, (b) day 3 and (c) day 6 of ESC culture show that the feeder cells sustain ESC growth and proliferation. (C) Optical images of the feeder cells cultured on the wells on (a) day 6, (b) day 8, and (c) day 11 show the formation of monolayer and that number of the cells remains unchanged over time. Scale bars = 100 μm.
The ESCs cultured on the feeder cells (Fig. 32A) display small cellular aggregates that increase in size with time and remain attached to the layer of feeder cells over time, exhibiting unique morphologies and growth characteristics of ESCs. In contrast, the ESCs cultured on the bare surface without feeder cells (Fig. 32B) show tiny spherical and isolated aggregates, which deteriorated over time. The results illustrate the significance of feeder cells that provide required proteins and scaffolds to sustain the proliferation of undifferentiated ESCs over time, and demonstrate that given PEFs are well suited for the production of viable growth-arrested feeder cells for culture of undifferentiated ESCs over time.

We further characterized the ESCs cultured on the feeder cells by detecting a widely used molecular marker of undifferentiated ESCs, membrane alkaline phosphatase (ALP), using an ALP staining assay. The dye-tagged antibody specifically binds with ALP on the cells, leading to a red-orange color. In contrast, no color was observed in the absence of ALP. As reported previously, ESCs express high levels of membrane ALP, which is down regulated upon the differentiation.

The ESCs cultured on the feeder cells and the bare surface without the feeder cells for 3 days, and the feeder cells alone were assayed using the ALP staining kits, as shown in Figures 33A - C, respectively. We observed the red-orange stains on ESC aggregates cultured on the feeder cells (Fig. 33A), and colorless for the cells cultured in the absence of the feeder cells (Fig. 33B) and feeder cells alone (Fig. 33C). The results show the presence of ALP on the ESCs cultured on the feeder cells, but an absence of detectable levels of ALP on the ESCs cultured without feeder cells or the feeder cells alone. Since the ALP staining assay required several washing and fixing steps, we observed a partial removal of some cellular aggregates (ESCs). Nonetheless, the red-orange stains of ALP on ESCs were unambiguous, which illustrates that ESCs cultured on the growth-arrested feeder cells prepared using PEFs, indeed remain undifferentiated over time. Taken together, the results demonstrated that the
Figure 33. Characterization of undifferentiated ESCs using the ALP staining assay.

(A) ESCs cultured on the growth-arrested feeder cells, (B) ESCs cultured on the bare surface without the feeder cells, and (C) growth-arrested feeder cells alone show red-orange colonies in (A) and colorless in (B, C). Images in (a – c) and (d – f) were acquired using digital color camera and CCD camera, respectively. Scale bars = 25 μm in (A), 50 μm in (B) and 100 μm in (C).
PEFs are well suitable for preparing viable growth-arrested feeder cells for culturing and sustaining undifferentiated ESCs over time.

**SUMMARY**

In summary, we have developed a new, simple, green, and effective technique (electric pulses) for producing viable and growth-arrested cells, and have used them as high-quality feeder cells to culture and sustain undifferentiated zebrafish ESCs over time. The PEFs can be generated using either a 10nsEP generator or a widely available inexpensive conventional electroporator, demonstrating the possibility of using freshly prepared feeder cells to culture and sustain undifferentiated ESCs in a wide variety of research laboratories for expansion and advance of ESC research. We found that the effects of PEFs on the cellular functions highly depend upon the duration, number and E of the pulses, suggesting that PEFs penetrate into the cells and inactivate mitotic processes. This study offers a powerful new tool for one to produce a wide variety of feeder cells for culture of various ESCs.

**METHODS**

**Reagents and supplies**

Reagents in this study were used as received and purchased from Sigma, except those indicated.

**Culture and assay of the cells (RTS34st)**

We received four 100% confluent flasks (25 cm²) of rainbow trout spleen cells (RTS34st), generously provided by Dr. Paul Collodi, and harvested the cells by trypsinization (2 mg/mL trypsin, 1 mM EDTA) and centrifugation (1915 rpm for 10 min).114, 121 We resuspended the cells in Leibovitz’s L-15 medium, and
determined their number and viability using a hemacytometer and Trypan blue assay, respectively.

**Utilizing PEFs to prepare growth-arrested feeder cells**

The RTS34st cells (500 μL, 1.0x10^6 cells/mL) were placed into each electroporation cuvette (Biosmith; distance between two electrodes in the cuvette = 0.4 cm). The same number of cells was used for control experiments. We exposed the cells to 25 sequential 10nsEPs of 150, 40 and 25 kV/cm (1-s pulse interval), provided by a 10-ns pulse generator (ARC Technology), as described previously.\(^{116}\) The 10nsEPs were measured in real-time using an oscilloscope (TDS 3052B, Tektronix).\(^{116}\) The cells in each cuvette were centrifuged at 1915 rpm for 5 min and rinsed with the L-15 medium twice via centrifugation. The same number of cells from each cuvette were finally seeded into flasks (25 cm²), and incubated at 19°C. After 30 min, 30% (v/v) heat-inactivated fetal bovine serum (FBS, GIBCO-BRL) was added into each flask.

Using the same approaches, we exposed the cells in each cuvette to two sequential 50μsEPs of 2.83, 1.78 and 0.75 kV/cm (5-s pulse interval), generated by a conventional electroporator (Bio-Rad, Gene Pulser Xcell). The cells were then seeded and cultured in the flasks, as described above. The cells were also seeded into each well of a 6-well culture plate (Greiner Bio-One). An additional 3 mL of L-15 medium was added to each well. The 6-well plate was sealed and incubated at 19°C for 30 min, then 30% FBS was added to each well.

We imaged the cells in the flasks and in the wells daily using optical microscopy with a 10x objective (bright-field upright Nikon E-400 or phase-contrast inverted Zeiss Axiovert, respectively). The microscopes were equipped with a high-resolution CCD camera (Micromax and CoolSNAP, Roper Scientific) and a high-definition color digital camera (Sony) to acquire images of the cells over time.\(^{52, 122}\) Twenty identical locations on the surface of each flask and well were marked, allowing us to image and monitor the cell growth in given areas over time. Note that RTS34st cells are adherent cells and only the cells attached
on the surface can grow over time. Once the feeder cells in each well became a confluent monolayer, the ESCs were transferred onto the wells and cultured over time.

We determined the cellular viability at the end of cell culture in the flasks and in the wells using Alamar blue viability assay (Invitrogen). We replaced the medium in each flask with fresh L-15 medium, and incubated them for 2 h. We then added Alamar blue dye (2% v/v) into the flasks and incubated them overnight. Photos of the flasks were taken over time.

**Isolation, culture and characterization of zebrafish embryonic stem cells**

We placed 10 wild-type mature females and 5 wild-type mature males (Aquatic Ecosystems) in a clean 5-gallon breeding tank and used a light (14 h) - dark (10 h) cycle to trigger breeding. For each experiment, we collected embryos at the blastula stage (approximately 4 hpf), transferred them to a Petri dish containing egg water (1.0 mM NaCl in DI water), and rinsed them thoroughly with egg water to remove any debris.

To isolate the ESCs, the embryos were soaked in 70% ethanol for 5 s, and then thoroughly rinsed with egg water and LDF medium. LDF medium was prepared by mixing L-15, Ham's F12, and Dulbecco's modified Eagle's medium at the ratio of 50:35:15 in 15 mM HEPES buffer containing 120 μg/mL penicillin G, 25 μg/mL ampicillin and 200 μg/mL streptomycin sulfate, with the addition of sodium bicarbonate (0.180 g/L) and sodium selenite (10^{-8} M). We removed the chorions from the embryos by first incubating the embryos with bleach solution (0.5% v/v) for 2 min and rinsing them with LDF medium immediately after each incubation and repeated four times. After the bleach rinses, the embryos were incubated in pronase-E solution (0.5 mg/mL in Hank's balanced salt solution) until the chorions started to break apart. We well rinsed the embryos with LDF medium and dissociated ESCs from the inner mass of embryos by incubating them with trypsin/EDTA (2 mg/mL trypsin, 1 mM EDTA)
for 2 min. We removed the trypsin/EDTA via centrifugation (1514 rpm for 5 min) and resuspended the ESCs in LDF medium.

We seeded the ESCs on the surface of the wells with and without a monolayer of the feeder cells and incubated the cells at 22°C over time. Each well contained 0.4 mL freshly prepared ESCs (3.0 x 10^5 cells/mL), 1.4 mL LDF medium, 5% FBS, 1% trout plasma (SeaGrow, East Coast Biologies), 1% bovine insulin, 1% human epidermal growth factor, 0.5% human basic fibroblast growth factor, 0.5% zebrafish embryo extract (10 mg/mL of proteins), and 31% RTS34st cell-conditioned medium, which was prepared by incubation of a confluent flask of RTS34st cells in L-15 medium containing 30% FBS for 3 days at 19°C and then filtered using 0.2 µm filters. The embryo extract was prepared by homogenizing ~500 embryos in 0.5 mL LDF medium, followed by centrifugation (10000 rpm for 10 min). The supernatant was collected and filtered using 0.2 µm filters. The concentration of proteins in embryo extract was determined using a biophotometer (Eppendorf). The extract was prepared freshly and stored at -20°C until use.

We imaged the ESCs in the wells daily using a phase-contrast inverted microscope equipped with a CCD camera and color digital camera. We used alkaline phosphatase (ALP) staining assay (Cell BioLabs Inc.) to characterize the undifferentiated ESCs over time. We first removed the medium from the wells cultured with ESCs and feeder cells alone, and washed the cells with PBST (1x PBS containing 0.05% Tween-20). The fixing solution (400 µL) of assay kit was added to each well and incubated at room temperature for 3 min. The cells were then washed twice with PBST. The staining solution (400 µL, freshly prepared by mixing equal volume of staining solution A and staining solution B of the assay kit) was added to each well of the culture plates, followed by incubation in the dark at room temperature for 30 min. The cells were washed twice with PBS and imaged using the phase-contrast optical microscopy.
Data analysis and statistics

At each given time, the cells (RTS34st or ESCs) on given areas (twenty selected surface locations) of the flasks or the wells were imaged using bright-field or phase-contrast optical microscopy. For ALP staining assay, the cells (ESCs or growth-arrested feeder cells) on given areas (twenty selected surface locations) of the flasks or the wells were imaged using bright-field optical microscopy. Each experiment was repeated at least six times.
CHAPTER VI

NEW METHODS, ELECTRIC PULSES, FOR CULTURING AND SUSTAINING EMBRYONIC STEM CELLS

INTRODUCTION

Embryonic stem cells (ESCs) are pluripotent cells that possess the unique ability to self-renew and the capability to differentiate into cell types found in all three germ layers. ESCs have the potential to provide a limitless source of functional cells for therapeutic applications, tissue replacement in regenerative medicine, drug discovery and toxicology, as well as important tools in cancer research. However, there are many challenges and questions that remain unknown that need to be addressed in order to effectively use ESCs in biomedical applications. One of the key challenges for researchers is to elucidate the mechanisms involved in cell lineage commitment and differentiation. Thus, understanding and controlling cell fate determination is necessary for the development of cell-based therapies. However, before establishing methods for controlling differentiation of ESCs, the mechanisms that maintain pluripotency and the techniques required for a successful large-scale culture and expansion of undifferentiated ESCs still needs to be addressed.

ESC cultures can be complex, where both ESC self-renewal and pluripotency are highly dependent upon feeder layers (FLs) and growth factors. FLs are composed of cells where DNA replication has ceased, while RNA and protein synthesis continues, hence the cells are mitotically inactive but remain viable. These cells are metabolically active to provide the ligands or cytokines needed for the proliferation of pluripotent ESCs.

Currently, applying gamma (γ) irradiation or treatment with mitomycin-C are the only two methods used to mitotically inactivate feeder cells. γ-irradiation causes DNA strand breaks which inhibits DNA replication,
whereupon, mitomycin-C forms cross-links between DNA opposite strands to inhibit DNA replication.\textsuperscript{32, 35} Nonetheless, there have been numerous studies comparing both methods, with each study concluding different results.\textsuperscript{34, 35, 123-125} One study indicated that mitomycin-C treatment reduced the viability and the metabolic activity of the feeder cells, hence reducing the availability of specific ligands or cytokines required for successful cell expansion.\textsuperscript{35} In contrast, another study suggested that mitomycin-C treated feeder cells maintained their adherent properties for 6 weeks and remained viable, while $\gamma$-irradiated feeder cells began to detach after 4 weeks, concluding that mitomycin-C is a cheaper and effective alternative to irradiation.\textsuperscript{34} Furthermore, using $\gamma$-irradiation is faster and less labor intensive than mitomycin-C treatment, which can be time-consuming, however the availability of a suitable radiation source can be problematic.\textsuperscript{112} Nonetheless, both studies demonstrated that feeder cells can differ in their vulnerability to both methods, indicating that the effects of each treatment should be assessed for each cell type used, as well as the need for a new and more effective method to prepare feeder cells.

In our previous study, we developed an innovative and efficient technique using 10-ns electric pulses (10nsEPs) and 50-\mu s electric pulses (50\mu sEPs) to produce viable and growth-arrested feeder cells to culture and sustain undifferentiated zebrafish ESCs.\textsuperscript{126} In this study, we characterized and compared the ability of feeder cells; (mouse embryonic fibroblasts) prepared using either 50\mu sEPs or $\gamma$-irradiation, to sustain mouse ESC (mESC) pluripotency throughout several passages and further differentiation into cardiomyocytes.

RESULTS AND DISCUSSION

PEFs generate viable growth-arrested feeder cells

We exposed mouse embryonic fibroblasts (CF-1), typically used as feeder cells for mouse and human ESCs, to two sequential 50\mu sEPs at 5, 10 and 15
kV/cm (5-s pulse interval). CF-1 cells without any exposure to PEFs and cells exposed to γ-irradiation were used to compare the effects of PEFs. The growth curve (Fig. 34a) of cells without any exposure to PEFs or γ-irradiation (Fig. 34A: a) shows the number of cells increased with time. Similarly, the number of cells exposed to a low (5 kV/cm) PEF also increased with time (Fig. 34B: a). The growth curves of cells exposed to either a 10 kV/cm PEF or γ-irradiation indicate that the number of cells remained constant with time (Figs. 34C: a and 34E: a, respectively). In contrast, the number of cells slightly decreased over time when exposed to a 15 kV/cm PEF (Fig. 34D: a). The representative optical images, shown in b - d (Figs. 34A - E), illustrate normal cell morphology and division (Figs. 34A - B), elongated and mitotically inactive cells (Figs. 34C and E), and cells with a small, spherical shape, indicative of unhealthy, apoptotic cells (Fig. 34D).

In order for cells to serve as effective feeder layers they must not only be growth-arrested but also remain viable and metabolically active.\textsuperscript{34-36} We tested these criteria using Alamar blue reagent. Healthy, viable cells maintain a reducing state within their cytoplasm, hence when Alamar blue, which is cell-permeable, enters the cell it is converted from its oxidized form (blue in color, $\lambda_{\text{max}}$ of absorption spectrum = 600 nm) to its reduced form (pink in color, $\lambda_{\text{max}}$ of absorption spectrum = 570 nm).\textsuperscript{118, 119} Therefore, Alamar blue can serve as a colorimetric assay to determine not only viability but metabolic activity as well.\textsuperscript{118, 119} After 5 days of culture we added Alamar blue reagent to wells (in triplicate) (Figs. 34F: a - e) containing CF-1 cells that weren't exposed to PEFs or γ-irradiation (Fig. 34F: a), cells exposed to a 5, 10 or 15 kV/cm PEF (Figs. 34F: b - d, respectively) and medium alone (Fig. 34F: e). The medium alone remained blue, while the unexposed cells and the cells exposed to a 5 or 10 kV/cm PEF turned from a blue to pink color, indicating they were viable after 5 days. Furthermore, the cells exposed to a 15 kV/cm PEF didn't remain blue in color but didn't completely change to pink either, indicating that the culture contained some alive and some dead cells. Taken together, these results indicate that
Figure 34. Effects of electric field strength and γ-irradiation on growth and viability of feeder cells.

(a) The growth curves and optical images on day (b) 1, (c) 3 and (d) 5 of cells that had been (A) unexposed and exposed to 2 sequential 50μsEPs (5-s pulse interval) at an E of (B) 5, (C) 10, (D) 15 kV/cm and exposed to (E) γ-irradiation. (F) Photo of 6-well culture plates containing Alamar Blue with: (a) unexposed cells at day 6 (control), (b - d) the cells at day 6 in (B - D), respectively, and (e) medium alone (blank control). Scale bars = 100 μm.
cellular growth and viability is dependent upon the electric field strength they are exposed to. Additionally, the two sequential 50μsEPs at 10 kV/cm generated viable, metabolically active and growth-arrested cells, when compared to γ-irradiated cells, had similar growth rates and cellular morphology.

**DNA content and viability of cells exposed to PEFs or γ-irradiation are quantitatively similar**

To further characterize and compare feeder cells exposed to PEFs or γ-irradiation, we quantitatively assessed both DNA content and viability. Representative images, taken over time, of unexposed cells and cells exposed to a 10 kV/cm PEF or γ-irradiation are shown in a - d in Figures 35A - C, respectively. The images of unexposed cells illustrate an increase in growth however once confluency is reached the cells start to die and detach from the culture vessel due to over-growth (Fig. 35A). In contrast, the cells exposed to a 10 kV/cm PEF (Fig. 35B) or γ-irradiation (Fig. 35C), growth remains constant, with no over-growth, and the cells remain attached.

To quantitatively determine whether the cells were mitotically inactivated we used a cell membrane permeable DNA stain to quantify the amount of cells in the different phases of the cell cycle. Analysis of DNA content is measured by the capacity of a DNA-binding dye to bind stoichiometrically to the double helix of DNA. Hence, the fluorescence emitted is directly proportional to the cell's DNA content. Additionally, the cell cycle phases: G0/G1 (resting, non-dividing cells), S (DNA is synthesized) and G2/M (process of mitosis) can be distinguished, where there is a lower DNA content in cells at the G0/G1-phase and a higher DNA content in cells at the G2/M-phase. The frequency distributions (Fig. 35D) represent the proportion of cells sorted according to their DNA content (fluorescence intensity). These results illustrate that the unexposed cells, from 24 - 72 h, were in all different phases of the cell cycle, indicating that the cells were actively dividing (Fig. 35D: a). In contrast, the cells exposed to a 10 kV/cm PEF or γ-irradiation (Figs. 35D: b – c, respectively) both showed the
Figure 35. Characterization of viable, growth-arrested feeder cells.

(A - C) Optical images of unexposed cells and cells exposed to γ-irradiation and a 10 kV/cm PEF, respectively, at (a) 24, (b) 48, (c) 72, and (d) 96 h after seeding. (D) Frequency distributions of the amount of DNA content, measured by fluorescence intensity, found in (a) unexposed cells and cells exposed to (b) γ-irradiation or (c) a 10 kV/cm PEF 24, 48, and 72 h after seeding. (E) Plots of viability using Alamar blue, measured as absorbance (OD<sub>570nm</sub>), vs. time for (a) unexposed cells and cells exposed to (b) γ-irradiation or (c) a 10 kV/cm PEF. Scale bars = 100 μm.
majority of cells, at 24 h, had a high amount of DNA content, indicating the cells were in the S or G_2/M-phases. However, after 24 h, the majority of the cells shifted to a lower DNA content, suggesting the cells where in the G_0/G_1-phase and remained there until 72 h. Thus, the cells exposed to PEFs or γ-irradiation were at the resting and non-dividing stage.

To quantitatively assess cellular viability and proliferation over time, we added the Alamar blue reagent to unexposed and exposed cells. The absorbance (OD\textsubscript{570nm}), which is proportional to the number of viable and proliferating cells, was determined at different time points. The plots (Fig. 35E) illustrate that the cells are proliferating and viable, for unexposed and exposed cells, until 30 h at which point, the number of proliferating and viable unexposed cells starts to steadily decrease. This decline in viable cells, as mentioned previously, is because of over-growth in the culture vessel (Fig. 35A). Furthermore, the number of proliferating and viable exposed cells, both to a 10 kV/cm PEF or γ-irradiation remained relatively constant throughout 96 h. The initial steady increase in proliferating and viable exposed cells coincides with the cell cycle phases determined from the amount of DNA content discussed above (Figs. 35D: b - c). These results indicate that cells exposed to a 10 kV/cm PEF or γ-irradiation contain similar growth characteristics and nuclear DNA content, while remaining viable over time.

**Generation of effective feeder layers for pluripotent mESCs**

We used the growth-arrested and viable feeder cells prepared using a 10 kV/cm PEF or γ-irradiation as a confluent FL of cells to culture mESCs (Figs. 36 and 37, respectively). The mESCs were subcultured three times every 72 h. Representative images taken every 24 h after the initial seeding onto feeder cells exposed to PEFs or γ-irradiation and after each subculture are shown as a – c in Figure 36A – D and Figure 37A – D, respectively. These images illustrate that the mESCs cultured on both types of exposed feeder cells are initially small, round colonies; however the cells become larger aggregates over time.
Figure 36. Characterization of undifferentiated mESCs cultured on growth-arrested feeder cells prepared using PEFs.

Optical images of mESCs (a) 24, (b) 48, and (c) 72 h after the (A) initial seeding onto FL cells, (B) 1st subculture, (C) 2nd subculture and (D) 3rd subculture. (E) Optical and corresponding (F) immunofluorescent images of undifferentiated ESC surface marker, SSEA-1, of representative mESC colonies after the (a - c) 1st, 2nd and 3rd subculture, respectively, indicating the mESCs remained undifferentiated. Scale bars = 100 μm in (A - D) and 250 μm in (E - F).
Figure 37. Characterization of undifferentiated mESCs cultured on growth-arrested feeder cells prepared using γ-irradiation.

Optical images of mESCs (a) 24, (b) 48, and (c) 72 h after the (A) initial seeding onto FL cells, (B) 1st subculture, (C) 2nd subculture and (D) 3rd subculture. (E) Optical and corresponding (F) immunofluorescent images of undifferentiated ESC surface marker, SSEA-1, of representative mESC colonies after the (a - c) 1st, 2nd and 3rd subculture, respectively, indicating the mESCs remained undifferentiated. Scale bars = 100 μm in (A - D) and 250 μm in (E - F).
We characterized pluripotency of the mESCs by using immunofluorescence staining to detect stage specific embryonic antigen-1 (SSEA-1), a common cell surface glycolipid that is downregulated upon differentiation in mESCs.\textsuperscript{110, 129} SSEA-1 staining was performed before each subculture to ensure the mESCs weren't differentiating. Representative optical and corresponding fluorescence images after the \textsuperscript{1\textsuperscript{st}}, \textsuperscript{2\textsuperscript{nd}} and \textsuperscript{3\textsuperscript{rd}} subculture, shown as a - c in Figures 36E - F and Figures 37E - F, illustrate that the cellular aggregates are staining positive for SSEA-1. These results indicate that mESCs can be routinely subcultured on feeder cells exposed to PEFs and remain undifferentiated after several passages. Furthermore, the results also show that mESCs cultured on feeder cells exposed to PEFs have similar proliferating and pluripotent characteristics to those cultured on feeder cells exposed to \textgamma-irradiation.

**Induced differentiation of mESCs cultured on feeder cells exposed to PEFs or \textgamma-irradiation**

To determine if the mESCs cultured on feeder cells exposed to PEFs, \textgamma-irradiation or with no FL, retained the ability to differentiate into different cell lineages, we induced differentiation into cardiomyocytes. Bone morphogenetic proteins (BMP), are proteins that regulate a variety of cellular functions that underlie cardiac differentiation and development.\textsuperscript{130} Hence, recent studies have used BMP4 as an inducer to investigate different processes occurring during cardiac differentiation.\textsuperscript{131-133}

To induce differentiation, the mESCs were removed from the feeder cells to form cell aggregates called embryoid bodies (EBs) through hanging drop, suspension and plating stages.\textsuperscript{134} The formation of EBs is required for \textit{in vitro} differentiation because during this process specific developmental signals that occur during normal embryogenesis are taking place.\textsuperscript{135} Additionally, the development of cardiomyocytes can be enhanced by the addition of supplements to the culture at specific time periods.\textsuperscript{135} Hence, BMP4 was added to serum-free
medium throughout the suspension stage.\textsuperscript{131-133} Once the EBs were plated, they were imaged every 24 h (Fig. 38). From the images, the EBs during mESC differentiation are initially a small and compact round cluster; however as differentiation time increases the cell number increases and after two weeks the colonies start to form a tissue-like appearance. Morphologically, we didn't observe any difference between the ESCs incubated with or without BMP4 cultured on $\gamma$-irradiated FLs (Figs. 38A - B), FLs exposed to a 10 kV/cm PEF (Figs. 38C - D), and those cultured on no FL (Figs. 38D - E).

Several days after plating the EBs, we characterized differentiation by performing immunofluorescence staining to detect the cell surface glycolipid, stage specific embryonic antigen-4 (SSEA-4). In contrast to SSEA-1, which is downregulated during differentiation, SSEA-4 is upregulated in differentiating mESCs.\textsuperscript{110, 136} The representative optical (Figs. 39: a and c) and corresponding fluorescence (Figs. 39: b and d) images of EBs cultured with (a - b) or without (c - d) BMP4 for mESCs cultured on feeder cells exposed to $\gamma$-irradiation, PEFs or no FL are shown in Figures 39A - C, respectively. These images illustrate that the EBs, in the presence or absence of BMP4, formed from mESCs cultured on feeder cells exposed to $\gamma$-irradiation, PEFs or no FL stained positive for SSEA-4, hence they are differentiating. However, there is a large amount of positively stained cells for SSEA-4 in those cultured on FLs and in the presence of BMP4 (Figs. 39A - B: a - b) than those cultured without a FL (Fig. 39C) or in the absence of BMP4 (Figs. 39: c - d). Furthermore, we quantitatively characterized the amount of SSEA-4 using the fluorescence intensity (Fig. 39D). The results confirm what was revealed in the images, that there is a higher number of differentiating cells in those cultured in the presence of BMP4. Additionally, the amount of differentiating cells was similar between the types of FL the mESCs were cultured on and no FL. These results indicate that mESCs cultured on feeder cells exposed to PEFs or $\gamma$-irradiation retain their ability to differentiate.
Figure 38. Formation of embryoid bodies (EBs) during differentiation of mESCs.

Representative optical images of EBs at (a) 1, (b) 3, (c) 5, (d) 7 and (e) 14 days after plating, derived from mESCs cultured on feeder cells exposed to either $\gamma$-irradiation in the (A) presence or (B) absence of BMP4, a 10 kV/cm PEF in the (C) presence or (D) absence of BMP4 or no FL in the (E) presence or (F) absence of BMP4. Scale bars = 150 $\mu$m.
Figure 39. Characterization of induced differentiation of mESCs.

Optical and corresponding immunofluorescent images of differentiation surface marker, SSEA-4, in mESCs that were cultured on feeder cells exposed to (A) γ-irradiation, (B) PEFs or (C) no FL with medium supplemented (a - b) with BMP4 and (c - d) without BMP4. (D) Plot of the average fluorescence intensity of SSEA-4 vs. type of FL (IRR or PEF) or no FL, and in the presence or absence of BMP4. Scale bars = 250 µm.
To determine whether differentiation was cell-lineage specific, we used immunofluorescence to detect cell surface biomarker, CXCR4. CXCR4 is a chemokine receptor that has different functions during embryonic heart development and can be used to identify cardiac progenitor cells. The representative optical (Figs. 40: a and c) and corresponding fluorescence (Figs. 40: b and d) images of EBs cultured in the presence (a - b) or absence (c - d) of BMP4 for mESCs cultured on feeder cells exposed to γ-irradiation, PEFs or no FL are shown in Figures 40A - C, respectively. These images illustrate that the EBs formed from mESCs cultured on feeder cells exposed to γ-irradiation (Figs. 40A: a - b) or PEFs (Figs. 40B: a - b), in the presence of BMP4, have a greater number of positively stained cells than those cultured without a FL (Fig. 40C) or in the absence of BMP4 (Figs. 40: c - d). Furthermore, using the fluorescence intensity of the cells, we could quantitatively characterize and determine that EBs from both types of feeder cells, in the presence of BMP4, had a much higher number of cardiomyocytes, than those cultured in the absence of BMP4 (Fig. 40D). Additionally, EBs formed from mESCs cultured without a FL had a much lower number of cardiomyocytes, regardless of whether BMP4 was added or not. Taken together, these results suggest that mESCs cultured on feeder cells, either exposed to PEFs or γ-irradiation, can form EBs and using a cell-lineage specific inducer, have the ability to differentiate into cardiomyocytes.

Furthermore, spontaneous contracting began 5, 11 and 12 days after plating the EBs, cultured in the presence of BMP4, derived from mESCs cultured on feeder cells exposed to γ-irradiation, PEFs or no FL, respectively. The EBs were observed daily, up to 27 days, the number of beating colonies (Fig. 41A) and the beating frequency (Fig. 41B) for each contracting area was determined. The percent of beating EBs vs. time (Fig. 41A), illustrate that the amount of beating EBs from mESCs cultured on feeder layers exposed to γ-irradiation, PEFs or no FL, gradually increased and then started to decline. The decrease in contracting EBs, is more than likely due to the fact that fully differentiated cardiomyocytes will often stop contracting, hence the reason we see the
Figure 40. Characterization of induced differentiation of mESCs into cardiomyocytes.

Optical and corresponding immunofluorescent images of a cardiac surface marker, CXCR4, in mESCs that were cultured on feeder cells exposed to (A) γ-irradiation, (B) PEFs, or (C) no FL with medium supplemented (a - b) with BMP4 and (c - d) without BMP4. (D) Plot of the average fluorescence intensity of CXCR4 vs. type of FL (IRR or PEF) or no FL, and in the presence or absence of BMP4. Scale bars = 250 μm.
Figure 41. Characterization of mESCs induction to differentiate into functional cardiomyocytes.

Plots of the (A) average percentage of beating EBs (total of 36 EBs plated) and (B) average beating frequencies per minute vs. time for EBs derived from mESCs cultured on feeder cells exposed to (◇) γ-irradiation, (♦) PEFs or (Δ) no FL.
the decrease in the amount of contracting EBs. A recent review on cardiomyocyte differentiation suggests that as EBs begin to spontaneously beat, the rate of contraction can change vigorously over a period of time. This explains why the average beating frequency per minute often varied and changed over time for each contracting EB (Fig. 41B). Taken together, these results confirm that EBs, cultured in the presence of BMP4, derived from mESCs cultured on feeder cells exposed to γ-irradiation, PEFs or no FL, can fully differentiate into functional cardiomyocytes.

SUMMARY

In summary, we employed our previously developed technique of exposing feeder cells to PEFs to generate a monolayer of growth-arrested feeder cells that are viable and metabolically active to culture mESCs. We found that the effects of PEFs is dependent upon the strength of the electric field and that cells exposed to a 10 kV/cm PEF are morphologically, metabolically and mitotically similar to cells exposed to a conventional method, γ-irradiation. Furthermore, mESCs cultured on feeder cells exposed to PEFs remain undifferentiated through several subcultures and have the ability to differentiate into functional cardiomyocytes. This study, along with our previous study, illustrate that PEFs can be used on different types of feeder cells that sustain various species of ESCs and are comparable to other techniques currently used. This technique offers a new and effective method for the successful long-term culture of pluripotent ESCs.
METHODS

Cell lines, reagents and supplies

Mouse embryonic fibroblasts (MEF) (CF-1), MEF (CF-1) γ-irradiated (IRR), mouse ESCs (J1), 1x Dulbecco’s modified Eagles medium (ES-DMEM and DMEM), fetal bovine serum (ES-FBS and FBS), 200 mM L-Alanyl-L-Glutamine in 0.85% NaCl, 10 mM MEM non-essential amino acids and phosphate buffered saline (PBS, without Ca²⁺ or Mg²⁺) were purchased from ATCC. 14.3 M 2-mercaptoethanol was purchased from Sigma and 10 μg/mL mouse leukemia inhibitory factor (LIF) was purchased from Chemicon. Cellular assays and stains, Alamar blue and Vybrant DyeCycle Orange were purchased from Invitrogen. For immunofluorescence, 10% goat serum, primary antibodies: anti-stage specific embryonic antigen-1 (SSEA-1) clone: MC-480, anti-stage specific embryonic antigen-4 (SSEA-4) and secondary antibodies: goat anti-mouse IgM FITC and goat anti-mouse IgG FITC were all purchased from Santa Cruz Biotechnology, Inc. For CXCR4 immunofluorescence, FITC rat anti-mouse CXCR4 was purchased from BD Biosciences.

Preparation of growth-arrested feeder cells

The MEF cells (CF-1) were first grown in culture flasks in DMEM with 15% FBS. When the cells reached 80% confluency, they were harvested by trypsinization and transferred (150 μL, 1.0 x 10⁶ cells/mL) to electroporation cuvettes (Biosmith; distance between two electrodes in the cuvette = 0.2 cm). We exposed the cells in each cuvette to two sequential 50-μs electric pulses of 5, 10 and 15 kV/cm (5-s pulse interval) using an electroporator (Bio-Rad, Gene Pulser Xcell). The cells from each cuvette were collected and centrifuged at 1200 rpm for 10 min and rinsed with culture medium twice via centrifugation. The cells (1.5 x 10⁵ cells/mL) were seeded onto 6-well culture plates (Griener Bio-One) and incubated at 37°C with 5% CO₂.
CF-1 (γ-irradiated) cells were thawed out according to product information and seeded (1.5 x 10^5 cells/mL) in culture plates, as well, in DMEM with 15% FBS and incubated at 37°C with 5% CO₂.

We imaged the cells in the plates every 24 h using optical microscopy with a 10x objective (phase-contrast, inverted Zeiss Axiovert). To image the cells over time, our microscope was equipped with a CCD camera (CoolSNAP, Roper Scientific) and a high-definition digital color camera (Sony). Twenty identical locations on the surface of each well were marked, enabling us to image and monitor the cell growth in a given area (1.2 mm²) over time.

We determined the viability of the cells using Alamar blue reagent. Briefly, we replaced the medium in each well with fresh medium and incubated for 2 h. We then added Alamar blue reagent (10%) to the wells and incubated them overnight. Digital photos of the well plates were taken over time.

**Quantitative characterization of growth-arrested feeder cells**

CF-1 cells were exposed to a 10 kV/cm PEF, as described above, and seeded (3.0 x 10^6 cells/mL) directly onto sterile coverslips in a 100 mm Petri dish. Growth medium was added to the dish to cover the coverslips. The same number of CF-1 (γ-irradiated) cells and control CF-1 cells (no exposure to PEFs or γ-irradiation) were added to coverslips in Petri dishes as well. All dishes were incubated overnight at 37°C with 5% CO₂. After 24 h, Alamar blue reagent (10%) was added to each dish. The dishes were then returned to incubate at 37°C with 5% CO₂. After 12 h, and periodically throughout 96 h, 50 μL of medium from each dish was removed to quantitatively determine the cellular viability, using UV-vis spectroscopy (Hitachi U-2010). The absorbance (OD₅₇₀nm) was measured for all samples, which is proportional to the viability and plotted with the reference subtracted.

Additionally, every 24 h, each dish was imaged and a cover slip from each dish was removed and thoroughly rinsed with PBS. 1.5 μM Vybrant DyeCycle
Orange was directly added to the coverslip and allowed to incubate at room temperature, in the dark, while gently shaking, for 1 h. The coverslip was then well rinsed with PBS and placed on a quartz microscope slide in a well-sealed microchamber. We then imaged the cells using epi-fluorescence microscopy (upright Nikon E-400), equipped with a CCD camera (Micromax, Roper Scientific) and fluorescence filter cubes (Chroma Tech) containing a band-pass excitation filter (488 ± 15 nm), band-pass emission filter (600 ± 15 nm), and a dichroic mirror (565 nm).

**Culture and assay of undifferentiated mESCs**

Mouse ESC line, J1, was used in this study. The mESCs (6.0 x 10⁴ cells/well) were cultured in 24-well plates on top of a monolayer of CF-1 FL (1.5 x 10⁵ cells/well) exposed to PEFs (10 kV/cm) or γ-irradiation in growth medium containing ES-DMEM supplemented with 2.0 mM L-Alanyl-L-Glutamine, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 1000 U/mL LIF and 15% ES-FBS. Growth medium was renewed daily. We imaged the mESCs colonies in the plates every 24 h and subcultured them every 72 h.

After each subculture the mESCs colonies were characterized for undifferentiation using pluripotent cell surface marker, SSEA-1. Briefly, growth medium was removed and each well was rinsed three times with PBS. The wells were then incubated for 1 h at 37°C with 5% CO₂ in 10% goat serum to prevent non-specific binding. After incubation, the goat serum was gently removed and each well was rinsed three times with PBS. Primary antibody, SSEA-1 MAB (1:400) in PBS was added to each well and incubated for 1 h and 30 mins at 37°C with 5% CO₂. At the end of the incubation time, the wells were rinsed three times with PBS and incubated with goat anti-mouse IgM FITC (1:100) in PBS as a secondary antibody for 1 h at 37°C with 5% CO₂. The wells were rinsed another three times with PBS and imaged using epi-fluorescence microscopy and fluorescence filter cubes containing a band-pass excitation filter (488 ± 15 nm), band-pass emission filter (525 ± 15 nm), and a dichroic mirror (500 nm).
Induction and assay of differentiated mESCs

The mESCs (6.0 x 10⁴ cells/well) were cultured in 24-well plates without a FL or on top of a monolayer of CF-1 feeders (1.5 x 10⁵ cells/well) exposed to PEFs (10 kV/cm) or γ-irradiation in growth medium containing ES-DMEM supplemented with 2.0 mM L-Alanyl-L-Glutamine, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 1000 U/mL LIF and 15% ES-FBS. Growth medium was renewed daily and the mESCs were subcultured every 72 h. Following the third subculture, mESCs colonies were harvested using 0.25% trypsin/0.53 mM EDTA and centrifugation (1200 rpm for 10 min). The mESCs were resuspended in ES-DMEM supplemented with 0.1 mM 2-mercaptoethanol, 2.0 mM L-Alanyl-L-Glutamine, 0.1 mM non-essential amino acids and 15% ES-FBS. To initiate differentiation, we performed the hanging drop method where the mESCs were pipetted into 20 µL drops, each drop containing approximately 800 mESCs, on the lid of a 100 mm tissue culture dish to produce embryoid bodies (EBs). The lid is inverted and placed over the dish containing PBS and incubated for 48 h at 37°C with 5% CO₂. After the 48 h incubation period, the EBs were transferred in ES-DMEM supplemented with 0.1 mM 2-mercaptoethanol, 2.0 mM L-Alanyl-L-Glutamine and 0.1 mM non-essential amino acids to a 60 mm bacteriological-grade Petri dish. Half of the dishes were additionally supplemented with 10 ng/mL BMP4, while the other half were not. All dishes were incubated for 120 h at 37°C with 5% CO₂. After the 120 h incubation period, the EBs were plated onto 0.1% gelatin-coated 24-well tissue culture plates (one EB per well) in ES-DMEM supplemented with 0.1 mM 2-mercaptoethanol, 2.0 mM L-Alanyl-L-Glutamine, 0.1 mM non-essential amino acids and 15% ES-FBS and incubated at 37°C with 5% CO₂. 24 h after plating, the medium is carefully removed and fresh ES-DMEM supplemented with 0.1 mM 2-mercaptoethanol, 2.0 mM L-Alanyl-L-Glutamine, and 0.1 mM non-essential amino acids is added to each well. All plates were incubated for at 37°C with 5% CO₂.
Five days after plating, characterization of differentiation was performed using SSEA-4 as a marker for determining loss of pluripotency.\textsuperscript{110, 136} Culture medium was removed from several of the wells that were treated with and without BMP4 and carefully washed three times with PBS. The wells were then incubated for 1 h at 37°C with 5% CO\textsubscript{2} in 10% goat serum to prevent non-specific binding. After incubation, the goat serum was gently removed and each well was rinsed three times with PBS. Primary antibody, SSEA-4 MAB (1:400) in PBS was added to each well and incubated for 1 h at 37°C with 5% CO\textsubscript{2}. At the end of the incubation time, the wells were rinsed three times with PBS and incubated with goat anti-mouse IgG FITC (1:100) in PBS as a secondary antibody for 1 h at 37°C with 5% CO\textsubscript{2}. The wells were rinsed another three times with PBS and imaged using epi-fluorescence microscopy and fluorescence filter cubes containing a band-pass excitation filter (488 ± 15 nm), band-pass emission filter (525 ± 15 nm), and a dichroic mirror (500 nm).

To determine cardiac-specific differentiation was occurring the EBs were characterized for CXCR4, which is a chemokine receptor suggested as a potential biomarker for cardiac progenitor cells.\textsuperscript{137} As performed previously, culture medium was removed from several of the wells that were treated with and without BMP4 and carefully washed 3 times with PBS. The wells were then incubated for 1 h at 37°C with 1% BSA in PBS as a blocking buffer to prevent non-specific binding. After incubation, the buffer was gently removed and each well was rinsed three times with PBS. FITC rat anti-mouse CXCR4 (1:150) in PBS was added to each well and incubated for 1 h at 37°C with 5% CO\textsubscript{2}. At the end of the incubation time, the wells were carefully rinsed three times with PBS and imaged using epi-fluorescence microscopy and fluorescence filter cubes containing a band-pass excitation filter (488 ± 15 nm), band-pass emission filter (525 ± 15 nm), and a dichroic mirror (500 nm).

The EBs were imaged every 24 h for 27 days using optical microscopy. A sequence of images of spontaneous contracting EBs was done and the beating frequency per minute was determined.
Data analysis and statistics

Growth rates for unexposed cells or cells exposed to PEFs or γ-irradiated were determined by counting 20 locations (1.2 mm²) for each time point, with averages and standard deviations presented. DNA content was determined by measuring the intensity per pixel (with background subtracted) using imaging software (Image J) for 100 cells at each time point for each cell type. The average intensity per pixel was measured (Image J) for SSEA-4 and CXCR4 fluorescent images for 50 cells per cell type, with averages and standard deviations presented. Each experiment was repeated 3 times.
CHAPTER VII

CONCLUSION

In summary, this dissertation presents the development of novel tools, ranging from biocompatible and photostable plasmonic Au and Ag NP imaging probes to ultrashort electric pulses, for the real-time study of embryonic development. We achieved the foundation necessary to potentially use NPs as \textit{in vitro} and \textit{in vivo} photostable optical molecular probes and sensors for the real-time study of important dynamic events occurring during embryonic development, including ESC differentiation.

In Chapter II, we synthesized and characterized highly purified and uniform 12 nm Au NPs to assess the transport, biocompatibility, and toxicity in a living \textit{in vivo} model system, zebrafish embryos, in real-time. We developed new imaging approaches using DFOMS-MSIS to characterize the transport of single NPs into developing zebrafish embryos. The NPs diffused into the chorion space and into the inner mass of the embryo non-invasively. It was observed that the diffusion rates of the Au NPs varied dramatically as it diffused through various parts of the developing embryo, suggesting high heterogeneity of embryonic nano-environments. Using DFOMS-MSIS, we found that the amount of Au NPs accumulated in the embryos increased with its concentration. Interestingly, it was observed that the Au NPs caused very little deformities and death that were not proportionally related to their concentration. This result is in stark contrast with what we reported previously for the same size Ag NPs, showing that Au NPs are much more biocompatible with the embryos than Ag NPs and suggesting that the biocompatibility and toxicity of nanoparticles depends on their chemical properties. These results suggest that zebrafish embryos can serve as an effective \textit{in vivo} nanotoxicity assay and that Au NPs can be utilized as a new biocompatible tool for single NP imaging of embryonic development in living embryos in real-time.
In Chapter III, we employed our *in vivo* model system used in Chapter II, and a larger size of purified and stable Au NPs (86.2 nm) to probe the size-dependent transport kinetics, biocompatibility and toxicity effects of NPs on single living embryos in real-time. Similar to the smaller size Au NPs, discussed in Chapter II, the NPs non-invasively diffused into the embryos via random diffusion and stayed inside the embryos throughout their entire development. It was observed that the chronic treatment of Au NPs caused majority of embryo developed to normal zebrafish, while an insignificant amount of embryos developed to deformed zebrafish or died. By comparing with our previous study of smaller Au NPs, in Chapter II, and various sized Ag NPs (11.6, 41.6 and 95.4 nm), we found the size-dependent biocompatibility of Au NPs and chemical-dependent biocompatibility and nanotoxicity of Au and Ag NPs. At the same given doses, the larger Au NPs are slightly more biocompatible with embryonic development than the smaller Au NPs, while the similar sized Ag NPs are much more toxic than the Au NPs. Interestingly, Ag NPs have a higher quantum yield than Au NPs, thus they illuminate much brighter and more colorful. Using our DFOMS-MSIS, Ag NPs are ideal for real-time multiplexing *in vivo* imaging. However, the results in Chapters II and III indicate Au NPs are much more biocompatible than Ag NPs, thus in order to use as *in vivo* imaging probes we would need to functionalize their surfaces with biocompatible peptides, as our previous study suggests. These results offer new understandings into the logical design of utilizing Au and Ag NPs as new biocompatible plasmonic imaging probes.

In Chapter IV, we synthesized and characterized purified and stable Ag NPs (97 nm) to probe the biocompatibility and toxicity of these Ag NPs upon specific zebrafish embryonic developmental stages in real-time. We found that the Ag NPs entered inside the embryos non-invasively and remained inside throughout their development upon their 2 h incubation with embryos at each given developmental stage. It was observed that upon acute exposure of a given stage embryo, the Ag NPs incite dose-dependent and stage-dependent effects.
We found that early developmental stages are more sensitive to the NPs than the later stages. Additionally, we observed distinctive phenotypes that resulted from the acute treatment at the earlier stages, indicating stage-specific effects of the Ag NPs. These results indicate that Ag NPs can target specific pathways in development and generate distinctive mutants for further identification of biomarkers for a better understanding of embryonic development.

In Chapters II, III and IV, we used zebrafish embryos as a model organism to study embryonic development and as an *in vivo* assay to assess the size- and chemical-dependent effects of Au and Ag NPs. The results offer new understandings into the design of more biocompatible plasmonic NP imaging probes for studying important processes occurring during embryonic development, including ESC differentiation, *in vivo* in real-time and for potential diagnosis and therapies for early embryonic development.

*In vitro* studies of ESC differentiation will set the groundwork for studying differentiation *in vivo*. However, the techniques required for a successful large-scale culture and expansion of pluripotent ESCs needs to be addressed first and foremost.

Thus, in Chapter V we developed a new, simple and effective tool to produce viable growth-arrested feeder cells for ESC culture. We used PEFs to generate high-quality feeder cells to culture and sustain undifferentiated zebrafish ESCs over time. We found that the cellular effects of PEFs depended directly upon the duration, number and electric field strength of the pulses. These results illustrated the possibility of altering the pulse variables to produce various types of growth-arrested cells for the proliferation of undifferentiated ESCs.

In Chapter VI, we further characterized our new tool, PEFs, developed in Chapter V, to prepare growth-arrested feeder cells for a different species of ESCs. We used PEFs generated from a conventional electroporator to produce viable high-quality feeder cells to culture and sustain undifferentiated mouse...
ESCs over time. Similar to Chapter V, we found that the cellular effects were dependent upon the electric field strength. Additionally, we found that over time the DNA content and cellular morphology of the feeder cells exposed to PEFs were similar to cells exposed to γ-irradiation, which is currently the most common method to prepare feeder cells. Most importantly, the results show that when mouse ESCs were cultured on feeder cells exposed to PEFs, they remain undifferentiated through multiple subcultures and were able to differentiate into functional cardiomyocytes. Notably, mouse ESCs are cultured on the same type of feeder cells as human ESCs. Thus, we can use our new tool to prepare growth-arrested feeder cells for the successful large-scale culture and expansion of human ESCs, which is the initial and most critical step in developing ESC-based therapies.

The results of the research mentioned in this dissertation have provided new and important information to the successful design of new tools for the study of embryonic development in real-time. We have demonstrated the ability to utilize living zebrafish embryos as a powerful in vivo model to use to study embryonic development and the real-time transport, biocompatibility, and toxicity of Au and Ag NPs. The potential applications to use NPs as new tools in biomedical research is vast, however their effects on living organisms remain largely unknown. The results in Chapters II – IV are the first step to developing NP probes for a variety of biomedical applications; including in vivo imaging, diagnosis, and ESC research. The results in Chapters V and VI conclusively show that our new tool, PEFs, generated using widely available inexpensive conventional electroporators, prepare different types of feeder cells to culture and sustain undifferentiated ESCs. This technique can be utilized in a wide variety of research laboratories for the expansion and advancement of ESC research. In summary, this dissertation has described the development of a wide range of innovative tools for real-time study of embryonic development and provided the groundwork necessary for the development of new ultrasensitive tools for the study of ESC differentiation both in vitro and in vivo.
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APPENDIX

GUIDELINES FOR HANDLING NANOMATERIALS

When using nanoparticles, standard laboratory practices regarding the use of hazardous chemicals are followed. The following work practice is required when handling nanoparticles to reduce potential exposure and ensure safe conditions in our laboratories. Lab coats must be worn or arm sleeves are required where high levels of exposure or splashes of solutions containing nanoparticles are anticipated. Eye protection, where appropriate to the tasks performed is required. This may include safety glasses, face shields, and/or chemical splash goggles. Gloves (disposable) are worn at all times when handling nanomaterials. Appropriate personal clothing is required in all laboratories. Long pants and closed toed shoes are required. Hand washing facilities are provided in all labs and hand washing must be performed after handling nanomaterials. All solutions are disposed of as hazardous waste following established University guidelines.
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